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CHARACTERISATION OF GLUTATHIONE S-TRANSFERASE-BASED DDT RESISTANCE IN *ANOPHELES ARABIENSIS*

Thesis submitted in accordance with the requirements of
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by

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DECLARATION

This work has not been previously accepted in substance for any other degree and is not being currently submitted in candidature for any other degree.

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ABSTRACT

Resistance to DDT in the mosquito vectors of malaria has seriously hampered efforts to control this disease and has contributed to a resurgence of malaria in recent years. In *An. gambiae* resistance to DDT is due to an increased metabolism of the insecticide by members of the glutathione S – transferase (GST) family of enzymes. Over expression of the class Epsilon GSTs is an important mechanism of resistance to DDT in this malaria vector.

Elevated GST activity has been detected in DDT resistant field population of the sibling species *An. arabiensis* but the molecular mechanism underlying this resistance is unknown.

In this study, colonies of the MAT and KGB laboratory strains of *An. arabiensis* were selected for low level resistance to DDT (×1.5) and (×2.2) respectively. The rates of early knockdown, KD 0 – 30 minutes and late KD 30 – 50 minutes, by permethrin were significantly lower in the DDT – selected ($p < 0.001$) and ($p = 0.072$) compared to the parental MAT population

Analysis of knockdown resistance *kdr* by PCR precluded the presence of a *kdr* mutation in the colonies and biochemical analysis suggests involvement of GSTs in resistance.

Degenerate primers based on conserved regions of *An. gambiae* Epsilon class *GSTe* gene cluster were used to amplify genomic and cDNA templates from *An. arabiensis*. A total of eight Epsilon class *GSTe1 – GSTe8* were isolated and sequenced. Blast results showed that *An. arabiensis* GSTs have a high sequence identity to *An. gambiae* (91% - 98%) at the amino acid level. Phylogenetic analysis supported the close taxonomic relationship between the two species.

The role of three of the GSTs, *GSTe1*, *GSTe2*, and *GSTe4* in DDT resistance was studied using quantitative real time PCR establishing the transcript levels of these genes in the developmental stages of the three strains of *An. arabiensis* which differed in their susceptibility to DDT.

The transcriptional activities of these Epsilon GSTs vary such that *GSTe1* and *GSTe2* are over expressed in the KGB – P and KGB – R strains compared to the MAT – P and *GSTe2* is over expressed in the DDT selected KGB- R line compared to parental KGB-P.

The expression of *GSTe2* was induced by hydrogen peroxide, permethrin and DDT exposure in both MAT and KGB strains.

Computational promoter analysis was performed on the promoter region of *GSTe1* and *GSTe2* from MAT, KGB and field strains of *An. arabiensis*. Basal putative regulatory elements and several other transcription sites were identified in the promoters for *GSTe1* and *GSTe2* in *An. arabiensis*. Further molecular studies are needed to confirm the functional role of these elements in the transcription of these genes.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
AhR	Aryl hydrocarbon receptor
AP-1	Activator Protein-1
ARE	Antioxidant-responsive element
AREB	Atp1a1 regulatory element binding factor
ATP	Adenosine tri-phosphate
bp	Base pair
BRE	TFIIB recognition element
CBF	CCAAT-binding factor
cDNA	Complementary DNA
CREB	Cyclic AMP Response Element-Binding
DEPC	Diethyl pyrocarbonate
DDE	1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane
DDT	1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane
DPT	Downstream promoter element
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosides triphosphate
dsDNA	Double-stranded DNA
DTT	Dithionthreitol
fg	Femtogram
FOXL1	Forkhead Box L1
GABA	Gamma-aminobutyric acid
gDNA	Genomic deoxyribonucleic acid
GPE	GST P enhancer
GRE	Glucototicoid responsive element
GSH	Glutathione
GST	Glutathione S-transferase
HNF-6	Hepatocyte Nuclear Factor
H_2O_2	Hydrogen peroxide
Inr	Initiator element
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITNs	Insecticide-treated nets
Kb	Kilobases
Kdr	Knockdown resistance
KDT	Knockdown Time
LB	Luria-Bertani
LD	Lethal dose
LDP	Log – dose probits
mg	microgram

M_gCL_2	Magnesium chloride
ml	Millitre
mM	Minimolar
mRNA	Message RNA
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NF-kb	Nuclear factor-kappaaB
NFAT	Nuclear factor of activated T cells
ng	Nanogram
NMP4	Nuclear matrix protein 4
OBF	Ocs binding protein
OP	Organophosphate
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus
rmp	Rotations per minute
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
Sox-5	Sry-type HMG box protein – 5
TBP	TATA box binding protein
TESS	Transcription Element Search Software
TLP	TATA box binding protein like factor
TRP	TATA box binding protein related factor
TSS	Transcription starting site
UTR	Untranslated region
µl	Microlitre
UPE	Upstream promoter element
v/v	Volume : volume ratio
w/v	Weight : volume ratio
WHO	World Health Organization
x-gal	5-bromo-4-chloro-3-indol-β-D-galactopyranoside
XRE	Xenobiotic responsive element

CHAPTER 1

GENERAL INTRODUCTION

1.0 General introduction

Insecticide resistance is a major obstacle in the control of insect pests of crops and vectors of animal and human diseases. As an example, in the1960s the World Health Organization (WHO) reverted from policy of malaria eradication to one of sustainable control, despite the initial success in eradicating malaria from some areas of its transmission by residual house spraying of DDT. This change in policy was necessitated largely because of the development of resistance in many Anopheline vectors of malaria (Hemingway *et al*, 2002). The Roll Back Malaria initiative campaign currently advocated and funded by WHO and other donors adopts the extensive use of pyrethroid - impregnated bednets for mosquito control campaigns in malaria endemic regions of Africa. However, there are already documented reports of pyrethroid-resistance in some populations of Anopheline malaria vector species from West, East , and South Africa (Martinez-Torres *et al*, 1998; Ranson *et al*, 2000a; Hargreaves *et al*, 2000). The operational impact of this resistance on malaria control is not clear (Curtis *et al*, 1998).

The slow rate of registration of new insecticides for malaria control, the absence of an effective malaria vaccine, and extensive drug resistance in the parasite may all worsen the threat posed by insecticide resistance to future malaria control efforts. These problems have prompted multidisciplinary efforts to achieve a better understanding of insecticide resistance mechanisms in the different Anopheline vector species (Phillips, 2001; Hemingway *et al*, 2002; Liu *et al,* 2006).

1.1 Control of Malaria Vectors

One approach to malaria control is to prevent the mosquito vectors from biting the human host. Mosquitoes are controlled by various methods using strategies that are directed against immature or adult stages. These programmes aim at reducing transmission of the disease by reducing the vector density through larval control or reducing the chances of adult mosquito survival to an age at where they can carry sporozoites and transmit disease (Curtis, 1996). Mosquito larval control has been attempted through various environmental management approaches, such as draining

or filling in of breeding sites, screening of water tanks to prevent mosquito breeding, or adopting a weekly intermittent irrigation schedule of breeding sites to interrupt the larval cycles (Dua, *et al,* 1988). The larvivorous fish *Ctenopharyngodon idella* have been used to control mosquito larvae in some part of Asia (Curtis, 1996). Similarly, the bacterial toxins from *Bacillus thuringiensis* are specifically lethal to mosquito larvae and have been used extensively against nuisance mosquitoes in Germany and USA. Anopheles mosquito larvae have been controlled by treating the breeding site with the organophosphate insecticide temephos (Dua et al, 1988). However, larval control can make a significant contribution to malaria control only where *Anopheles* breeding is sufficiently limited in extent and definable.

Impregnation of bednets

Nets have long been appreciated as a protection against night-biting mosquitoes including malaria vectors. However, nets are often torn or hung in such a way that mosquitoes can enter or bite through them. The motive for impregnating them with an insecticide, which is safe for close human contact, was to add a chemical barrier to the imperfect physical barrier presented by the net. In addition, the treated bed nets are a rational place in which to apply a residual insecticide because mosquitoes are attracted to them by the carbon dioxide and body odour emitted by the sleeper (Curtis, 1996). The pyrethroids are the only class of insecticides licensed for impregnation of bednets (Curtis, 1990; Miller, 1994). Insecticide treated bednets (ITNs) have been used successfully in malaria control programmes in China (Chen, *et al*, 1995) and in the Gambia (West Africa) (D'Allessandro, *et al*, 1995) and are advocated in the WHO Roll Back Malaria initiative for malaria endemic areas (WHO, 2000). However, it has been cautioned that large scale use of pyrethroid impregnated nets may select for pyrethroid resistance or may change mosquito behaviour so that they bite out of doors before people go indoors to bed (Curtis, 1996).

DDT and indoor residual house spraying

The most effective approach to prevention of malaria is generally to attack the adult *Anopheles* vector mosquitoes in houses where most of them bite and rest. The main method of attacking adult mosquitoes in houses is by spraying the inside surfaces of the walls and roof or ceiling with a residual insecticide such as DDT.

In many field studies, DDT showed spectacular repellent, irritant, and toxic actions that worked against malaria vector mosquitoes (Taverne, 1999). When DDT was sprayed on walls inside houses ($2g/m^2$) it exerted powerful control over indoor transmission of malaria. Malaria control by house spraying with DDT has produced excellent and rapid results in the USA, Italy, Venezuela, Guyana, India, and elsewhere (Robert, *et al*, 1999). There are reliable data showing the impact on vector-borne diseases of extensive use of DDT and in several cases, the resurgence in malaria that has followed its partial or complete withdrawal (see Table 1) (Curtis and Lines, 2000). During the 1960's and 1970's, small scale field trials and pilot campaigns of house spraying in equatorial Africa using DDT or other organochlorines were very successful. In the islands of Zanzibar and Pemba, DDT spraying reduced the prevalence of malaria to <50% (Kouznetsov, 1977). However, recently there has been an increasing trend in malaria cases globally. In South America, the population at high to moderate risk of malaria more than doubled in Colombia and Peru from 1996 to 1997 (Robert, *et al*, 2000). Malaria is reappearing in urban areas and in countries that previously eradicated the disease, e.g. North and South Korea (Feighner, *et al*, 1998). The current global increase in malaria cases has been contributed to by many factors, but the strongest correlation is with decreasing number of houses sprayed with DDT (Robert, *et al*, 1997). The abandonment of house spraying by DDT in Madagascar has resulted in an explosive malaria epidemic which was controlled by immediate restoration of DDT indoor spraying (Curtis, 2002a).

Table 1. Summary of history of DDT and vector borne disease in seven countries

Country	Pre-DDT	Active use of DDT	DDT use restricted or eliminated
India	c. 75 million malaria cases and 0.8 million malaria deaths per year; also many cases of VL[a]	In 1960s c. 18000 tonnes of DDT used annually; malaria down to c. 100000 cases, VL eliminated	7500 tonnes of DDT for use against malaria and VL in 1999–2000, >3 million malaria cases, VL reappeared
Sri Lanka	2–3 million cases and 80000 deaths in 1934–1935 epidemic	DDT campaign reduced number of reported cases to 17 in 1963	c. 360000 cases reported in 1994; DDT resistance – replaced by organophosphates and pyrethroids
USSR and successor states	Malaria as far north as Moscow and across southern Siberia, c. 3 million cases in 1940	Malaria virtually eradicated in 1950s and 1960s by DDT, bio-environmental control and case finding and treatment	c. 15000 cases in Tadjikistan and in Azerbaijan in 1996 and return of transmission in Ukraine and Urals
Italy	Marsh draining and quinine reduced number of malaria cases to 55000 in 1939	Malaria eradicated by a few rounds of DDT spraying in late 1940s	Eradication of transmission maintained despite many imported cases
South Africa	c. 22000 malaria deaths in 1931–1932; malaria morbidity paralysed the sugar industry	DDT spraying from late 1940s until 1990s drove malaria back to frontier regions without evolution of resistance in vector	Switch to pyrethroids in mid-1990s, malaria still in frontier regions (c. 7000 cases and 30 deaths per year)
Madagascar	Malaria endemic in lowlands, epidemics in highlands since 1878	DDT in 1950s eradicated malaria in highlands; DDT use re-started in 1990s to bring malaria back under control	Spraying stopped in 1960s; *Anopheles funestus* population recovered and in 1988–1991 caused an epidemic which killed many thousands
Venezuela[b]	One million cases per year; malaria death rate up to 80 per 1000 during epidemics – higher than that caused by 1918 influenza epidemic	Malaria eradicated from developed parts of the country (eg. Sucre state) by DDT spraying in 1940s, 1950s and 1960s	c. 24000 cases per year recorded in late 1990s (including c. 5000 in Sucre state), despite pyrethroid spraying

[a] Abbreviation: VL, visceral leishmaniasis.
[b] Experience of Venezuela apparently matched by six other Latin American countries, in contrast to Ecuador where DDT use has increased and incidence has declined[9].

Table 1.1: Summary of use of DDT and vector borne disease in seven countries (After Curtis and Lines 2000)

Resistance to DDT

Resistance slowly appeared in the 1960's in response to intensive agricultural use of DDT, especially in cotton production. The current distribution of DDT resistance among malaria vectors covers a small area of Columbia in South America (*An. darlingi*), limited regions located in south-east Asia, Iran, Pakistan, India, Sri Lanka (*An. culicifacies*), Greece (*An. sacharovi*), Egypt (*An. pharoensis*), West Africa (*An. gambiae*), and South Africa (*An. arabiensis*) (Mouchet, 1988; Suarez, *et al*, 1990; Hargreaves, *et al*, 2003).

1.2 Categories of insecticide resistance mechanisms

Resistance is defined as an inherited ability to tolerate a dosage of insecticide that would prove lethal to the majority of individuals in a normal wild (susceptible) population of the same species (WHO, 1981). Four categories of insecticide resistance mechanisms have been outlined. These are behavioural, physical, site-insensitivity, and metabolic resistance types (Miller, 1988). In general, site-

insensitivity and metabolic resistance are the main resistance mechanisms in insects and physiological resistance occurs as a result of interplay between these mechanisms (Oppenoorth, 1984).

1.2.1 Target Site Resistance

Most common insecticides produce their effects by acting on the insect nervous system. Target-site resistance develops when the chemical site of action for the insecticide is modified resulting in reduced sensitivity to the active form of the insecticide (Miller, 1988). The three central targets for conventional insecticides are the acetylcholinesterases, ligand-gated and voltage-gated ion channels. The biochemistry, molecular biology and mechanisms by which target-site resistance develops in several insect species have been comprehensively reviewed (ffrench-Constant, 1998; Hemingway *et al*, 2004; Liu *et al*, 2006).

Insensitive acetylcholinesterase

The organophosphates and carbamates target the enzyme acetylcholinesterase (AChE), which catalyzes the hydrolysis of the neurotransmitter acetylcholine on the post-synaptic nerve membrane. The insecticides inhibit enzyme activity by covalently phosphorylating or carbamylating the serine residue within the active site gorge (Corbett, 1974). Alterations in AChE in insects which are resistant to organophosphates and carbamates result in a decrease in sensitivity to inhibition of the enzyme by these insecticides (Ayad and Georghiou, 1975). This broad-spectrum resistance mechanism occurs in *An. albimanus* from Central America (Ayad and Georghiou, 1975; Hemingway and Georghiou, 1983) and Mexico (Penilla *et al*, 1998) and in *C. p. pipiens*, *C. p. quinquefasciatus*, *C. tritaeniorhnchus*, *An. nigerrimus*, *An. atroparvus* and *An. sacharovi* (Villani and Hemingway, 1987; Bisset *et al*., 1990; Hemingway *et al*, 1985, 1986). Resistance to the carbamate insecticides propoxur and carbosulfan, also due to insensitive AChE, has been described in *An. gambiae* (N'Guessan *et al*, 2003). In *Drosophila* only one gene encoding AChE, *ace* (Fournier *et al*, 1989) has been reported, and various mutations have been described in resistant strains (Mutero *et al*, 1994). The presence of at least two AChE genes in *C. p. pipiens* and *C. p. quinquefasciatus* was inferred from the findings that AChE-based resistance mapped to chromosome II, whereas the

cloned AChE gene (*ace-2*) is sex-linked (Bourguet *et al*, 1996; Malcolm *et al*, 1998; Mori *et al*., 2001). Subsequently, the sequences of two AChE genes (*ace-1* and *ace-2*) were identified from the *An. gambiae* genome (Holt *et al*, 2002).

GABA receptor

The type A receptor for the neurotransmitter γ-aminobutyric acid (GABA) is the target site of cyclodiene insecticides such as dieldrin. Binding of GABA to the receptor elicits rapid gating of an integral chloride-selective ion channel. GABA receptors comprise five sub units arranged around the central ion channel. Mutations at a single codon in the Rdl (resistance to dieldrin) gene encoding one receptor subunit, have been documented in all dieldrin-resistant insect species including *Drosophila melanogester*, *Musca domestica*, and *Aedes aegypti* (Hosie *et al* 1997; ffrench-Constant *et al*, 1998; Thomas *et al*, 1993). Fipronil, the phenylpyrazole insecticide also targets the GABA receptor and problems of resistance due to existing mutation may impact on the use of this insecticide in vector control programmes (Kolaczinski and Curtis, 2001).

Voltage-gated sodium channel

The insect voltage-sensitive sodium channels are the primary targets for pyrethroid insecticides and DDT. The sodium-channel protein is a symmetrical structure comprising four homologous domains (I-IV), each consisting of six α-helical transmembrane segments (S1-S6) (ffrench-Constant *et al*, 1998) and the channels serve as special "gates" within the membranes that separate nerve cells from the extra-cellular fluid (Tan *et al*, 2002). When an impulse passes along a nerve cell, the sodium channels open and close allowing an influx of sodium ions thereby depolarizing the membrane and causing an action potential. The action potential is then completely inactivated within a few milliseconds causing repolarization of the membrane (Liu *et al*, 2004).

Both pyrethroid insecticides and DDT operate by altering the function of the channels in the nerve membrane of insects, preventing the repolarization phase of the action potential (Narashi, 1996). Insects are known to develop resistance to pyrethroids and DDT through structural modifications of their sodium channels that reduce their sensitivity to insecticides. The term "knockdown resistance" (*kdr*) is

used to describe cases of resistance to DDT and pyrethroid insecticides in insects and other arthropods resulting from the reduced sensitivity of the sodium channels. Knockdown resistance was first characterised and studied in the house fly, *Musca domestica*. A substitution from leucine to phenylalanine at amino acid residue 1014 (L1014 F) resulting from a single nucleotide polymorphism termed the *kdr* mutation, in domain II segment 6 of the sodium channel (Williamson *et al*, 1993; Knipple *et al,* 1994) was first linked to moderate (10-30 fold) knockdown resistance in house fly strains (Williamson *et al*, 1996). An additional mutation of methionine to threonine at residue 918 (M918 T), termed the super-*kdr* mutation, was found to cause much higher resistance in house flies (Williamson *et al*, 1996; Miyazaki *et al*., 1996). These findings have inspired the search for similar mutations in other insect species and the *kdr* mutation has been subsequently demonstrated in association with resistance to pyrethroids and DDT in many insect species (Soderlund and Knipple, 2003).

The L1014F *kdr* mutation is prevalent in many insect species (Hemingway *et al,* 2004). In mosquitoes, this mutation is found in pyrethroid-resistant West African *An. gambiae* (Martinez-Torres *et al*, 1998), *An. stephensi* (Enyati *et al*, 2003), A*n. sacharovi* (Luleyap *et al*, 2002), *Culex pipiens* (Martinez-Torres *et al*, 1999; McAbee *et al*, 2004) and *Culex quinquefasciatus* (Xu *et al*, 2005). An alternative substitution of leucine to serine (L1014S) has been detected in pyrethroid-resistant *Culex pipiens* (Martinez-Torres *et al*, 1999), East African *An. gambiae* (Ranson *et al*, 2000a) and *An. sacharovi* (Luleyap *et al*, 2002). Heterozygotes containing both mutations have been found *An. gambiae* in West Africa (Etang *et al*, 2006; Pinto *et al*, 2006). Although several studies have documented high frequencies of the *kdr* alleles in *An. gambiae* s-s, surprisingly these are very rare in the sibling species *An. arabiensis* (Diabete *et al*, 2004; Verhaeghen *et al*, 2006; Kulkarni *et al*, 2006).

1.2.2 Metabolic resistance

This occurs when the metabolic pathways of insects become modified in ways that increase detoxification of insecticide, or disallow the metabolism of the applied compound to its toxic forms (Miller, 1988).Three enzyme systems, the esterases, monooxygenases and glutathione S- transferases (GSTs) are primarily involved in metabolic resistance.

Esterase-based resistance

B-type esterases have a high affinity for organophosphate insecticides. The enzymes rapidly sequester these insecticides and hydrolyse them and carbamates at very slow rates (Hemingway and Karunaratne, 1998). Elevated levels of esterases can prevent the insecticide from binding to its target site (Hemingway, 1982). The elevation of one or more non-specific esterases can result in very high levels of resistance to organophosphates in many insect pest species (Devonshire, 1976; Mouches, *et al*, 1986). This resistance mechanism has been demonstrated in the Culex species including *Culex pipiens pipiens*, *C. quinquefasciatus* and *C. tritaeniorhynchus* (Hemingway and Karunaratne, 1998). In most cases elevated esterase levels are due to extensive genomic amplification of the esterase genes (Vaughan, *et al*, 1995), although up-regulated transcription without an underlying gene amplification has been reported (Rooker, *et al*, 1996). The co-amplified esterases (*est2-2*^{1} and *est* β *2*^{1}), are the most common resistance genotype in *C. quinquefasciatus* (Vaughan, *et al*, 1997). In *Anopheles* mosquitoes enhanced esterase activity has been reported in *An. albimamus* (Brogdon and Barber, 1990) and permethrin-resistant *An. gambiae* (Vulule, *et al,* 1999). Malathion specific carboxylesterase resistance has been found in *An. culicifacies*, *An. stephensi* and *An. arabiensis* but the underlying genetic changes are not known in these mosquitoes (Herath, *et al*, 1988; Hemingway, 1983).

Monooxygenase-based resistance

Cytochrome P450-dependent monooxygenases are a large complex family of hydrophobic, heme containing enzymes involved in the metabolism of xenobiotics (Hemingway and Ranson, 2000). These enzymes have been implicated in the metabolism of most common insecticides.

The detoxification involves the introduction of oxygen into the insecticide substrate by the P450 enzymes (Brogdon, *et al*, 1997; Scott, 1999). Elevated P450 mono oxygenase activities, often in conjunction with altered activities of other enzymes, have been reported in insecticide-resistant mosquitoes. Brogdon *et al*, (1999) have reported both oxidase-based and esterase-based resistance mechanisms in permethrin-resistant *An. albimamus* from Guatemala. Vulule *et al*, (1999) demonstrated elevated oxidase and esterase levels in permethrin-resistant *An.*

gambiae from Kenya. Increased activity of P450 monooxygenases has been associated with permethrin-resistance in *An. funestus* from South Africa (Brook, *et al*, 2001). Most insects have very extensive families of P450s, but only a small subset is involved in insecticide metabolism. The *Cyp6d1* gene is over-produced in pyrethroid-resistant *M. domestica* due to up-regulated transcription (Kasai and Scott, 2000). Up-regulation of *Cyp6gl* orthologs are linked with DDT resistance in field isolates of Drosophila *melanogaster* (Daborn, *et al*, 2002). Laboratory selection of *D. melanogester* with DDT also results in up-regulation of *Cyp6g*l (at times in conjunction with *Cyp12dl*) or *CyP6a8* (Brandt, *et al,* 2002; Le Goff, *et al*, 2003). Analysis of genome data revealed a total of 111 P450 monooxygenases in *An. gambiae* (Ranson, *et al*, 2002). Nikou, *et al*., (2003) have recently shown elevated transcripts levels of an adult-specific *CypP6z1* gene in a pyrethroid-resistant strain of *An. gambiae*.

1.2.2.3 Glutahione S-transferases (GSTs) – based resistance

GSTs are a family of enzymes involved in metabolic resistance and are specifically important in DDT metabolism in insects hence they will be discussed in the following separate section.

1.3 Glutathione S- transferases (GSTs)

1.3.1 Structure and functions of GSTs

The insect GSTs exist in multiple forms exhibiting a wide range of substrate specificity with enzyme groups showing distinctive kinetic properties and class – specific expression profiles (Ranson and Hemingway 2005). Most of the GSTs in insects are soluble dimeric proteins found in the cytosol. The microsomal class which form the minority of the GSTs are membrane bound trimeric proteins. A cytosolic GST is a dimer of two subunits ranging from 24 to 28 KDa in size. Each subunit consists of an N-terminal and a C-terminal domain joined by a linker peptide chain. The N-terminal domain approximates to 1 – 80 residues consists of four β-sheets with three flanking α- helices, forming a thioredoxin-like fold which occurs in most proteins that bind to GSH (Sheehan *et al.,* 2001). The N-terminal domain contributes the majority of the amino acid residues to the G-site which binds

glutathione. A more variable substrate binding site, named the H-site, is located at the C-terminal (Mannervik, 1985,). One amino acid residue in the G site is primarily responsible for activating glutathione. In the majority of mammalian GSTs and in all Sigma GSTs, this active site residue is tyrosine, serine performs this function in the Zeta, Delta and Epsilon classes, while in Omega class GSTs cysteine is the residue at the active site (Sheehan *et al*, 2001). Studies on the 3 – dimensional structures of GST subunits have revealed additional residues that interact with glutathione (Agianian *et al,* 2003).

The diversity of the H site of GSTs relates to the various functions they perform in biological systems. The involvement of GSTs in enzymatic detoxification of both endogeneous and xenobiotic compounds is well established (Wilce and Parker, 1994; Armstrong, 1991). They also catalyse reactions in metabolic pathways associated with oxidative stress, thus protecting cells from damage (Sawicki *et al.*, 2003). In addition to their catalytic activities, GSTs may act as intracellular transporters of vital compounds including bilirubin, heme, thyroid steroid hormones and bile salts (Mannervik, 1985). However, GSTs in insects are studied particularly for their role in the detoxification of wide range of insecticides (see Section 1.3.4).

1.3.2 Nomenclature and classification

The nomenclature and classification of GSTs is a continuously evolving process as scientists acquire new knowledge on, and gain insights into, the structure and functions of these complex enzymes. The present classification system is based on various criteria including substrate specificity, nucleotide sequence similarity, type of the amino acid at the active site, immunological identity and tertiary structural properties. The use of these criteria to classify the mammalian GSTs and its implication on classification of GSTs from other sources has been reviewed (Sheehan *et al*, 2001). Five GSTs which were initially identified based on assumed substrate specificities from the rat liver, were named: GSH – aryltransferase, GSH – epoxidetransferase , GSH – alkyltransferase, GSH – aralkyltransferase and GSH – alkene – transferase (Mannervik,1985). However, this nomenclature soon proved to be inadequate and had to be modified to accommodate overlapping substrate specificity among enzymes, and the simultaneous existence of protein subunits as

both homo and heterodimers (Mannervik, 1985). Roman letters were used to name the transferases isolated from rat with reference to the order of their elution from a CM – celluse ion exchanger (Habig *et al,* 1974). Subsequently enzymes were given names to reflect the composition of their subunits and Arabic numerals were used for each variant subunit (Mannervik, 1985). In classifying the mammalian GSTs, combinations of the criteria mentioned above have been employed, but by consensus, GSTs that share greater than 60% sequence identity are placed in the same class, and less than 30% in a separate class (Sheehan *et al*, 2001). Eight classes of cytosolic GSTs that are of high similarity in amino acid sequences are recognized in mammals: Alpha, Mu, Pi (Mannervik *et al*, 1992), Sigma (Buetler and Eaton 1992), Theta (Meyer *et al*, 1991), Kappa (Pemble *et al*, 1996), Zeta (Board *et al*, 1997) and Omega (Board *et al*, 2000). The Kappa GST are unique in many features, the most important of which is the lack of the SNAIL/TRAIL motif found in all other GST classes (Sheehan *et al*, 2001).

In *An. gambiae,* twenty – eight encoding cytosolic GSTs were identified from the genome sequence (Ranson *et al*, 2002). These are categorized into six different classes. Two of which, the Delta and Epsilon class GSTs, appear to be specific to insects, containing 15 and 8 GSTs respectively. Of the remaining five *An. gambiae* GSTs, two genes, *GSTt1* and *GSTt2*, were classified in the Theta class (Ranson *et al*, 2002), which was earlier proposed to be the progenitor class of all GSTs (Pemble *et al*, 1992). The ubiquitous Omega, Zeta and Sigma classes (Board *et al*, 2000) are each represented by a single gene in *An. gambiae*. With the exception of Sigma class, all of the non – insect specific classes are expanded in *D. melanogaster* relative to *An. gambiae* (Ding *et al*, 2003).

The Epsilon class GST cluster in *An. gambiae* is formed of eight genes *GSTe1* – *GSTe8*. All the eight members of the *An. gambiae* Epsilon class are arranged sequentially on the right arm of chromosome 3 division 33 C .The cytological position of the *An. gambiae GSTe* genes is illustrated in Figure 1.1

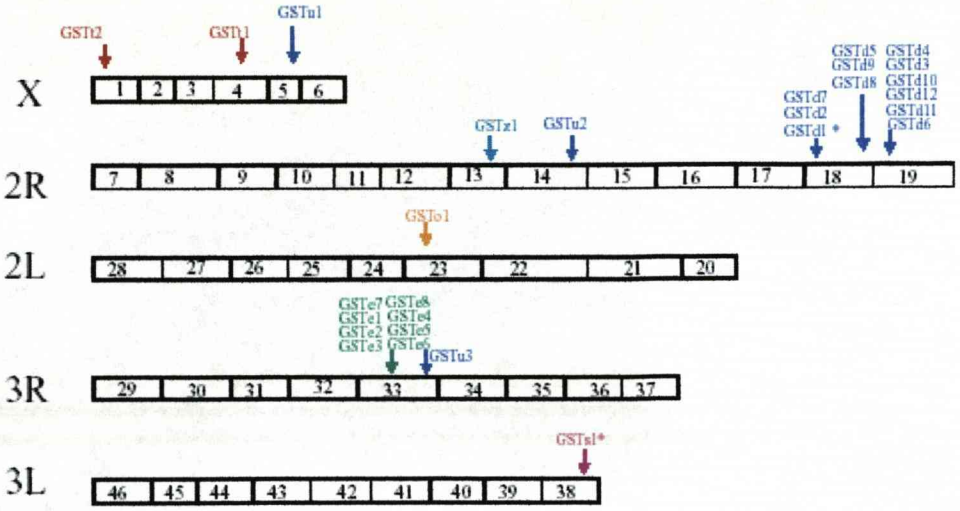


Figure 1.1: Schematic diagram indicating the organisation of GST genes in the *An. gambiae* genome.

The numbers represent polytene chromosome divisions. Those genes marked * are alternatively spliced to produce multiple transcripts. (From Ding *et al*, 2003)

The Epsilon GSTs cluster within approximately 11 kb of DNA. The length of each Epsilon GST cDNA ranges from 654bp to 693bp. The length of the intergenic space between neighbouring GST genes varies from 175bp to 1300bp. The transcriptional orientation of two Epsilon GST genes, *GSTe7* and *GSTe6*, are opposite to the remaining GSTs (Figure 1.2)

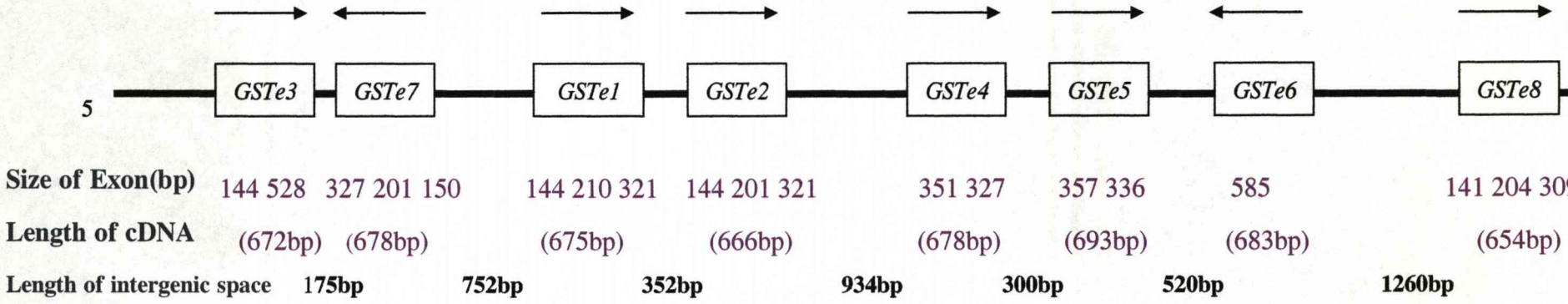


Figure 1.2: Genomic organization of the *A. gambiae* Epsilon GST genes.
Eight Epsilon GSTs allocated sequentially on chromosome 3. The length of Epsilon GST cDNA and the size of exons are indicated. Arrows represent the transcriptional orientation of Epsilon GST gene.

1.3.3 Role of GSTs in insecticide resistance

GSTs have been implicated in resistance to all the major classes of insecticides (Vontas, *et al*, 2001; Prapanthadara, *et al*, 1993; Huang, *et al*, 1998). Metabolic studies on multiple organophosphorous resistant strains of *An. subpictus* found that resistance to fenitrothion was correlated with an increase in activity of GSTs that detoxified the oxon analogue of the insecticide (Hemingway, *et al*, 1991).

An Epsilon class GST from the diamond back moth, *Plutella xylostella*, is involved in organophosphate resistance (Huang, *et al*, 1998). Detoxification of the organophosphorous insecticides occurs either by glutathione conjugation with O-alkyl portion of the insecticide or with the "leaving group", the latter regarded as an O-dearylation reaction (Chiang and Sun, 1993). In houseflies, GST-based organophosphate resistance involved conjugation of reduced glutathione to the parental insecticide or to its primary toxic analogue (Syvanen, *et al*, 1994). GSTs have not been involved directly in metabolism of pyrethroid insecticides, but they are elevated in pyrethroid-resistant insects. Elevated GSTs with a peroxidase activity conferred resistance to pyrethroids in a laboratory selected strain of *Nilapavata lugens* (Vontas, *et al*, 2001; 2002).

The GSTs were believed to serve in a non-catalytic capacity as binding proteins removing the oxygen free radicals generated through the action of insecticides. GSTs may also protect against pyrethroid toxicity in insects by sequestering the insecticide (Kastaropoulos, *et al*, 2001). GSTs are known to inactivate the toxic products of oxygen metabolism, the reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals and hydrogen peroxide (H_2O_2) (Knight, 1998), which are generated in the various pathways, or due to the effects of pollutants and chemicals such as insecticides (Cross and Jones, 1991). Some GSTs posses GSH peroxidase activity and others can catalyse the reduction of cellular peroxidases, mainly fatty acids and hydroperoxidases (Sandeep, *et al*, 2004). Peroxidase activity has been detected using the substrate cumen hydroperoxidase in insect GSTs from the Sigma, Delta and Epsilon classes (Vontas, *et al*, 2001; Singh, *et al*, 2001; Ortelli, *et al*, 2003; Lumjuan, *et al*, 2005).

1.3.4 GST-based DDT resistance in *An. gambiae*

Insect GSTs catalyze the dehydrochlorination of DDT. In the housefly the reaction proceeds via a base abstraction of hydrogen, catalysed by the thiolate ion generated in the active site of the GST, leading to the elimination of chlorine from DDT to produce DDE (Clarke and Shamaan, 1984).

Increased rates of DDT dehydrochlorination have been reported in a DDT-resistant strain of *Ae. aegypti* (Grant, *et al*, 1991), and a DDT resistant strain of *An. gambiae*, ZAN/U from Zanzibar, had elevated DDT dehydrochlorinase activity compared to a susceptible strain (Prapanthadara, *et al*, 1993). A combination of genetic mapping approaches, gene expression analysis and characterization of recombinant proteins have identified the Epsilon class of GSTs as playing the major role in conferring DDT resistance in *An. gambiae* (Ranson, *et al*, 2000b; 2001). Two quantitative trait loci (QTL) conferring DDT resistance in this species were identified, one of which rtdl is located between division 32C and 34C of chromosome 3 and coincides with cytological location of the class Epsilon GSTs. Hence, it was proposed that class Epsilon GSTs are the major enzyme family conferring resistance to DDT in the ZAN/U strain of *An. gambiae* (Ranson, *et al*, 2001). Five members of the Epsilon class gene cluster were over-expressed in a DDT resistant strain of *An. gambiae*, thus providing additional evidence for their role in resistance. One of these, *GSTe2* was over-expressed approximately 5-fold in the resistant strain and exhibiting higher DDT dehydrochlorinase activity (Ortelli, *et al*, 2003).

Further support for the role of *GSTe2* in DDT resistance was provided by recombinant *GSTe2* expressed *in vitro*. The recombination enzyme had DDT dehydrochlorination activity and the conversion of DDT to DDE was dependent on the amount of enzyme (Ranson, *et al*, 2001). The second QTL conferring DDT resistance named rtd2, physically mapped between division 21 and 22 of the chromosome 2 and did not coincide with the location of any GST genes in *An. gambiae.*

1.3.5 Tissue and developmental stage expression of GSTs

Expressions of GSTs vary considerably in different tissues and developmental stages in insects. Tissue expression of some GST isoenzymes has been demonstrated in *Ae. aegypti* in which *GST-2* was over-expressed in the head, thorax and abdomen but not in ovaries (Grant, *et al*, 1991). In the house fly, the Sigma class GST is predominantly localized on thin filaments of the indirect flight muscles (Franciosa and Berge, 1995) where it interacts with troponin-H presumably playing a structural rather than catalytic role (Clayton, *et al,* 1998). Many studies have shown that the GST activity varies widely during the development of insects (Grant and Matsamura, 1988; Kastaropoulos and Papadopoulos, 1998). In *Ae. aegypti*, GST activity increases during larval and pupal development, reaches a maximum in the newly emerged adult and decreases thereafter (Hazelton and Lang, 1983). The Epsilon class *GSTe2* is expressed at higher levels in the later developmental stages in this mosquito species (Lumjuan, *et al*, 2005). The majority of individual insect GSTs are expressed constitutively in all life-stages. In *An. gambiae*, transcripts for all but one of the GST super gene family have been detected in one-day old adult mosquitoes (Ding, *et al*, 2003). Recently, a microarray approach was used to demonstrate the developmental expression patterns of detoxification enzymes including the GSTs in *An. gambiae* (Strode, *et al*, 2006). The expression of the two Epsilon class *GSTe1* and *GSTe2* were higher in larvae than in adult and pupae stages while *GSTe4* was highly more expressed in pupae (Strode, *et al*, 2006).

1.3.6 Transcriptional regulation of GSTs

The expression of GSTs is controlled at transcriptional level, but the molecular mechanisms regulating the transcription in insects are not fully understood. Transcriptional regulation is effected by cis-acting DNA sequences that direct the assembly of the protein machinery responsible for transcription and these regulatory sequences are highly conserved in eukaryotic promoters (see reviews by Harshman and James, 1998; Arnosti, 2003). The regulatory region in eukaryotes, comprises four distinct control elements; a basal promoter, upstream promoter elements (UPE), cis-acting elements which regulate the transcription of gene, and enhancers or silencers located either distant or adjacent to the promoter, which can increase or repress the transcriptional activity of the gene in either orientation.

A basal promoter consists of about 100 bp of sequence surrounding the transcription initiation site that comes into close contact with the general transcription machinery. In some promoters, this region contains TATA sequences (consensus TATA AA) centered at -30, Initiator (Inr) sequences at +1 (consensus TCAGT), and down-stream promoter elements (DPE) at 30 (consensus A/GGA/TC/TGT) (Arnosti, 2003). The elements of the basal promoter provide nucleation sites for binding by basal transcription machinery. The TATA sequence interacts with the TATA binding protein (TBP), a crucial part of the basal transcription machinery that helps anchor the RNA polymerase and basal transcription factors at the promoter. TBP is a subunit of the multi component TFIID general transcription factor, which contains about 10 TBP-associated proteins (TAFS) (Aoyagi and Wasserman, 2000). The TBP is bound in a polar manner to the asymmetrical TATA sequences, TATAAAAG and TATAAAA (Davidson, 2003). The binding of TBP leads to the assembly of a properly oriented pre-initiation complex which contains RNA polymerase II and other general factors, thus influencing the direction of transcription. After the binding of TFIID to the DNA, a second transcription factor TFIIB, joins the transcription complex by binding to TFIID (Drapkin, *et al*, 1993). The TFIIB acts as a bridging factor allowing the recruitment of RNA polymerase to the initiation complex. In genes that do not contain a TATA box, the TBP is recruited to the promoter by another transcription factor, which binds to the initiator element. The binding of TBP to initiator-binding protein allows the recruitment of TFIIB and RNA polymerase II to form a stable initiation complex. The initiator element, therefore, plays an essential role in the assembly of initiation complex (Kutach and Kadonaga, 2000). In addition to the TATA box and initiator, the downstream promoter element (DPE) identified in promoters that lack a TATA box motif, functions with the initiator for the binding of TFIID in the transcription of core promoters in the absence of TATA box (Burke and Kadonaga, 1996). The transcription activity of an initiation complex can be elevated by interaction with upstream promoter elements (UPEs) and their specific regulatory sequences to increase basal transcription levels (Jones, *et al*, 1998). Transcriptional regulation of genes is also effected by trans-acting elements that function by interacting with their recognised DNA sequences (see review by Kadonaga, 2002). Gene transcription can be regulated by many *trans*-acting elements and the binding of each *trans*-acting element to a *cis*-element has a different effect on transcription. The binding of

trans-acting elements to specific *cis*-elements can increase or repress the transcription level, the final output of which is a combined effect of the multiple transcription factors in response to physiological or environmental stimuli.

1.3.7 Induction of GSTs

GSTs are induced in insects by xenobiotics such as barbiturates and various insecticides including parathion, methyl paraoxon, carbaryl, dieldrin and DDT (Yu, 1996; Hayaoka and Dautermann, 1982). Induction of expression of GSTs in response to xenobiotic compounds is mediated through different transcriptional mechanisms about which little is known. Analysis of mammalian GST promoters has revealed the presence of several regulatory elements. Xenobiotic response elements (XRE) found in the flanking region of rat GST gene, are activated by planar aromatic compounds and may induce transcription of the rat *GSTA1* gene (Rushmore and Pickett, 1993). Similarly, the antioxidant responsive element (ARE), a second *cis*-acting regulatory element that also responds to planar aromatic compounds, is found flanking the rat *GST A1* gene (Frilling, *et al*, 1992; Prestera, *et al*, 1993). The promoter region of the rat *GSTA2* gene (*rGST A2*) contains XRE, which is responsible for both basal and inducible transcription of the gene (Telakowski-Hopkins, *et al,* 1988). The treatment of transfected cells with beta-NF, a compound that can bind with AhR increased the transcriptional activity of *rGSTA2,* suggesting the role of XRE in up-regulating the induced expression of GSTs by aryl hydrocarbon. Many GST subunits are inducible by phenobarbiturates via the *cis*-acting elements termed the Barbie Box (Liang, *et al*, 1995). The consensus sequence in Barbie Box contain in many eukaryotic genes is 5'-ATCAAAAGCTGGAGG-3'. The 4 bp sequence in the consensus fragment 5'-AAAG-3' is considered a core element of Barbie Box and conserved in all barbiturate-inducible genes. NF-Kb, a *trans*-acting element is responsive to H_2O_2 and other oxidative-stress related agents (Thanos and Maniatis, 1995). Putative, NF-Kb binding sites have been identified in 5' flanking region in *An. gambiae* Epsilon class *GSTe2* which was over-expressed by treatment with H_2O_2 (Ding, *et al*, 2005).

1.4 Detection of Insecticide Resistance

Insecticide resistance in mosquitoes can be detected using the WHO standard methods and in some cases the resistance mechanism involved can be identified by biochemical and molecular approaches (Brown and Brogdon, 1987; Hemingway and Ranson, 2000).

1.4.1 WHO susceptibility bioassays

The WHO has developed standard bioassay tests to detect insecticide resistance in the laboratory and field populations of mosquitoes in larval and adult stages (WHO 1975). In the WHO standard test, mosquito larvae in lots of 20-25 are exposed for 24 hours to the test insecticide in water at four different concentrations. About 300 larvae in the third or early fourths instar are required for the complete test. The WHO test kit provides standard solution of the principal larvicides in ethanol, prepared so that aliquots of 1ml of the standard added to 250ml water give the appropriate range of concentrations in mg/litre (ppm) to be used for the test. The concentration for which a standard is provided is five times higher than the preceding one, thus giving a (5-fold) interval. This range of concentration is sufficient to obtain test mortalities above 0% and less than 100% for any population sample of larvae, whatever its susceptibility level. The dosage-mortality figures obtained are plotted on graph paper relating the dosage on a logarithmic scale to the percentage mortality (probit) scale, and a regression line is drawn to fit these points (WHO, 1981). From this line the susceptibility level may be read off in terms of the LC50 and LC90, in concentrations in mg/litre (ppm) which are expected to cause 50% and 90% mortalities respectively. The results obtained with the population tested are then compared with the base-line figures for that species obtained from a normal susceptible population.

WHO (1986) has proposed standard tentative diagnostic dosages to detect resistance in mosquito larvae in the field populations. Survival of any larvae from exposure to the diagnostic dosages would indicate the possibility of resistance among the population tested. Similarly, diagnostic doses for adulticides have been determined by exposing susceptible one-day old non-blood fed female mosquitoes to a series of insecticide concentrations. This has allowed the determination of the lethal dose to

kill 99% (LD99) of the test population (Brown 1986). The diagnostic dose recommended by WHO is set at twice the LD99 for the least susceptible *Anopheles* species. Adults in batches of 20-25 mosquitoes are exposed to a test tube containing a paper impregnated with a diagnostic concentration of an insecticide for 60 minutes. Mortality is determined after 24 hours holding period. The protocol recommends using a minimum number of 100 mosquitoes to be tested at given concentration of exposure times with replicates of 20-25 mosquitoes (WHO, 1998). The WHO (1998) has provided standard test kits including papers impregnated with diagnostic concentrations for the known insecticides belonging to the organophosphate, carbamate, pyrethroid and organochlorine classes. For instance, the diagnostic dose of DDT determined for different anopheline species including *An. arabiensis* is 4% DDT for a 1 hour exposure. Following exposure of adult anophelines to diagnostic doses, the evaluation criteria suggested by Davidson and Zahar, (1973) and adopted by WHO are: 98% mortality indicates susceptibility, 80-98% requires verification and 80% suggests resistance (WHO, 1998). The bioassay approach is simple, inexpensive and has been described as the best resistance detection technology available for use in the field (Brown and Brogdon 1987). Nevertheless, the outcome of the susceptibility test depends on the prevailing environmental conditions and the physiological state of the insect at the time of the test. Variables such as high temperature or nutritional status which influence the behaviour of the insect can produce misleading results (WHO, 1998). Additional limitations to the use of bioassays are their inability to detect the underlying mechanisms of resistance and the requirement for many insects (Brogdon, 1989).

1.4.2 Biochemical assays

The need to address the practical problems which were associated with WHO bioassays has stimulated research efforts which led to the development of biochemical assays that were designed to detect resistance in the field. Appropriate use of biochemical and immunological methods for detecting resistance can provide a powerful tool for analysing field and laboratory population (Brown and Brogdon, 1987; Brogdon 1989). The biochemical assays are used not only to establish the underlying mechanism involved in resistance but also to measure changes in resistance gene frequencies in field populations under different selection pressures

(Penilla et al. 1998). Two variants of biochemical assays, the 'filter' paper or nitrocellulose membrane and the microtitre plate assays have been developed for use in detection of insecticide resistance. The assay method using solid support media such as "filter" paper generally uses one mosquito per assay and can be quantified visually or by densitometer (Hemingway, 1998). These assays provide a permanent record which can be rechecked in the future. The microtitre plate tests allow the same insect to be used for all assays and are quantified visually or with a spectrophotometer.

A description of the equipment, solutions and reagents required to run microtitre assays specific for altered acetylcholinesterase, elevated esterase, monooxygenase, glutathione S-transferase and protein have been explained in detail (Hemingway, 1998). The general equipment needed to conduct these assays includes microtitre plates, homogenizers, microcentrifuge tubes, microfuge pipettes and a microtitre plate reader. Various solutions of sodium monophosphate and sodium biphosphate at stated pH and molar concentrations are also required for the assay of the individual enzyme system. An individual mosquito is homogenised in distilled water in flat-bottomed microtitre plates on ice. The homogenisation can be done with the use of a 96-pin Teflon homogeniser that matched with the 96 wells of the microtitre plate. Aliqots of the homogenate are used in assays to detect altered AchE, GST, esterase and P450 levels. An example of the microtitre plate procedure for esterase assay using the substrates alpha and beta naphthyl acetate is illustrated in Figure 1.3. Individuals having non-elevated levels of esterase activity should have a pale blue or pink colour with alpha or beta naphthyl acetate respectively. Individuals with elevated esterase activity show an intense blue/black or pink/red colour with alpha or beta respectively. The advantages of the biochemical methods are that many of the assays involve a colour change that can be monitored visually, although it is more accurately measured spectrophotometrically (Brogdon, 1989). In addition, the reaction can be undertaken in a microtitre plate or on filter paper and a single mosquito homogenate is adequate for many tests. Nevertheless, there is no simple field biochemical assay for all resistance mechanisms.

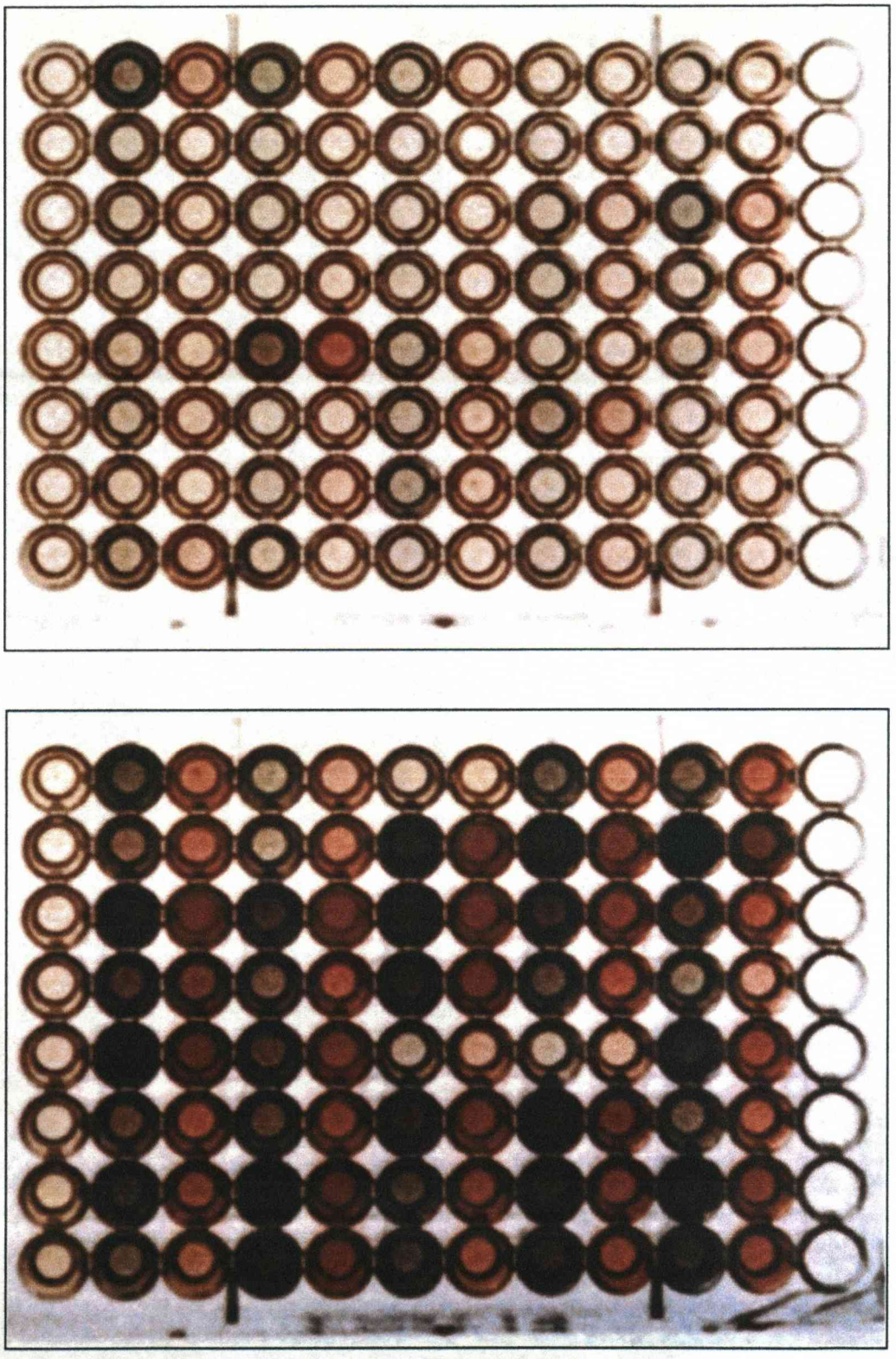


Figure 1.3: Two microtitre plates showing the esterase assays with alpha (blue) and (red) napthyl acetate
Column 1 is controls without homogenate. The upper plate is fully susceptible strain of mosquito. The lower plate is mixed resistant population with resistant mosquito showing the dark red coloration.
(after Hemingway , 1998)

1.4.3 Molecular assay

In order to use molecular assays to detect insecticide resistance the nature of the mutations resulting in resistance must first be determined. At present there are no molecular assays to detect metabolic resistance. The only resistant mechanism that can be detected by molecular assay are those for target-site insensitivity such as the mutations which confer *kdr*. To date, more than 20 unique sodium channel amino acid sequence polymorphisms have now been identified as being involved in reducing sodium channel sensitivity to insecticides (Hemingway *et al*, 2004). This type of resistance can be detected by PCR approach (Martinez-Torres *et al*, 1998; Ranson *et al*, 2000a; Tripet *et al*, 2006).

Martinez-Torres *et al*, (1998) have developed a simple diagnostic PCR assay that enables rapid diagnosis of the common leucine to phenylalanine (TTA to TTT) substitution. This method has been used to identify resistant and susceptible *kdr* alleles in West African populations of *An. gambiae*. The technique allows detection of heterozygotes in field populations. The PCR *kdr*-diagnostic assay uses primers AgD1 and AgD2 that flank the region containing the *kdr* mutation and amplify a 293 bp product from genomic DNA. Primers AgD3 and AgD4, internal to this region, are allele specific. Primer AgD3 binds only to the resistant *kdr* allele and when paired with AgD1, will amplify a 195 bp fragment if this allele is present in the individual. AgD4 binds only to the susceptible allele and will pair with AgD2 to produce a 137 bp band if the susceptible allele is present. This PCR method was modified to develop a diagnostic assay specific for detection of leucine to serine mutation (Ranson *et al*, 2000). Figure 1.4 schematically illustrates the design of the diagnostic PCR methods for the two mutations. The PCR methods have been commonly used to detect the presence of the *kdr* allele and estimate its frequency from large numbers of individuals. However, the very low amplification efficiency of the PCR precludes the analyses of DNA samples of low concentration or of poor quality. Also, because of high rates of null-alleles in the four priming sequences, estimates of the frequency of the *kdr* allele in wild populations are likely to be inaccurate. In certain instances some samples cannot be amplified at all. Another approach that was used is a simple PCR amplification followed by sequence-specific

oligonucleotide probing (PCR-SSOP) (Kolaczinski *et al*, 2000) but the additional hybridization steps limit the extensive use of this method.

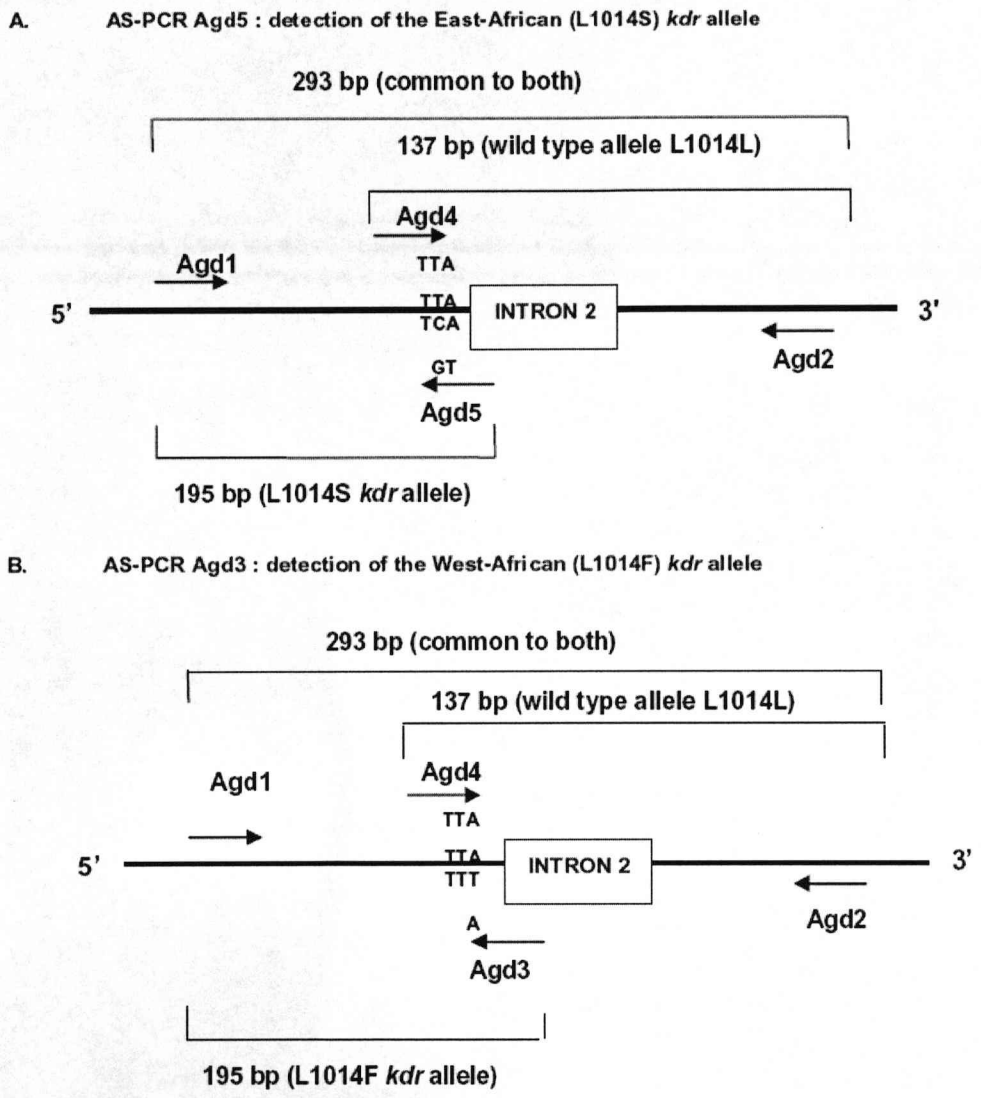


Figure 1.4: Schematic representation of the AS – PCR Agd5 and AS – PCR Agd3.
Schematic representation of the AS – PCR Agd5, which detects the L1014S *kdr* allele(A) and the AS – PCR Agd3, which detects the L1014F *kdr* allele (B) (from Verhaeghen *et al*, 2006)

An alternative approach based on a hot ligation oligonucleotide assay (HOLA) has also been developed that allows for convenient scoring by visualisation of a fluorescein-labelled reporter after allele-specific ligation to a detector (Lynd *et al*, 2005). Although this approach has the advantage of requiring no electrophoresis equipment, it is more complex and time consuming than the multiplex PCR. A

similar diagnostic PCR that uses fluorescent primers and enables simple detection of the *kdr* allele has recently been used to diagnose both the leucine to phenylalanine and leucine to serine substitutions in *An. gambiae* (Tripet *et al*, 2006). Very recently, Kulkarni *et al*, (2006), have described a new method, sequence-specific oligonucleotide probes (SSOP-ELISA) that was used for simultaneous detection of both East and West Africa *kdr* mutations. The method uses PCR and enzyme-linked immunosorbent assay (ELISA)-based technology allowing rapid testing of large numbers of individual mosquitoes. This method was used to detect presence of leucine to phenylalanine *Kdr* mutation in *An. arabiensis* populations in Tanzania (Kulkarni *et al*, 2006).

1.5 The *Anopheles gambiae* complex

In tropical Africa, the intensity of malaria transmission is sustained by the presence of the two efficient vector groups, the *An. funestus* and *An. gambiae* complexes (Gillies and Coetzee, 1987). The latter consists of seven sibling species namely: *An. melas* Theobald, *An. merus* Donitz, *An. gambiae* (Giles, *An. arabiensis* Patton, *An. quadriannulatus* sp.A Theobald, *An. quadriannulatus sp.B* Hunt, Coetzee, Fettene and *An. bwambae* White. Further complexity is added by the process of incipient speciation within *An. gambiae* s.s (Coluzzi *et al*, 1985). Reproductive incompatibility, expressed often as hybrid male sterility, exists between all the sibling species when ever they are crossed in the laboratory and this has been interpreted as indication for existence of reproductive isolation in nature (Davidson and Jackson, 1962, Davidson, 1964; Paterson *et al,* 1963). Cytogenetic studies based on the examination of the polytene chromosomes of *Anopheles* mosquitoes provided evidence for the taxonomic values of the chromosomal banding patterns and inversions which can be used to separate the sibling species of the *Anopheles gambiae* complex (Coluzzi and Sabatini, 1967, 1968; Davidson and Hunt, 1973). The polytene chromosome differences found among the species of the complex consist essentially of changes in the band sequences due to the inversions in the regions of the chromosomes. Ten inversions which are found only as inverted homozygotes in natural populations are fixed in the different species and there are a number of species – specific polymorphic inversions (Coluzzi et al. 1979). A schematic map showing the typical arrangements of the inversions on the

chromosomes of each species has been worked out diligently and is used as a key for the identification of the sibling species (Coluzzi et al. 1979).

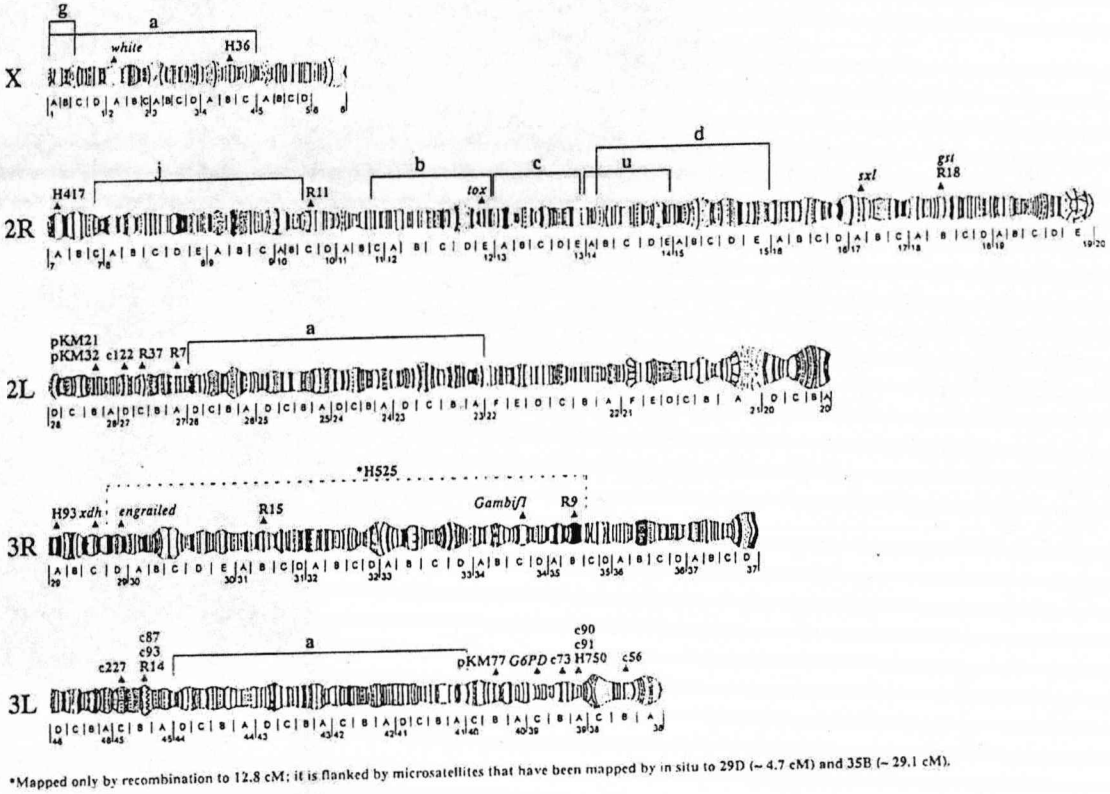
Recently, additional barriers to gene flow within West African population of *An. gambiae s.s* has been speculated based on lack of expected intergradations between alternative arrangements of polymorphic inversions. This phenomenon, termed as "incipient speciation", led to the separation of *An. gambiae s.s* into reproductive units or incipient species designated as Forest, Bissau, Bamako, Savanna, and Mopti (Toure *et al*, 1998). The Mopti and Savanna + Bamako chromosomal forms correspond to the M and S molecular forms in Mali and Burkina Faso (Della Torre *et al*, 2001). Mosquitoes of the S form are characterized mainly by inversion polymorphisms typical of the Savannah and Bamako chromosomal forms while M show chromosome – 2 arrangement typical of the Mopti (Della Torre et al 2001). However, both the M and S molecular forms have the standard karyotype associated with the Forest chromosomal forms of *An. gambiae* in Cameroon (Wondji *et al*, 2002).

The sibling species of *An. gambiae* complex are now identified accurately and more efficiently using DNA probes and PCR (Collins *et al*, 1988; Scott *et al,* 1993). Based on the species – specific nucleotide sequences in this region, a PCR method using five 20 base olignucleotide primers is routinely employed to identify single specimens of the *An. gambiae* complex (Scott *et al*, 1993). PCR methods can also be used to detect the chromosomal forms of the incipient species of *An. gambiae s.s.* (Favia *et al,* 1997; Fanello *et al*, 2002).

1.5.1 *Anopheles arabiensis* Patton

It is important to distinguish the sibling species of the *An. gambiae* complex as different species exhibit differences in their ecology, vectorial capacity and response to control programmes (Coluzzi et al 1979). *An. arabiensis* is the most closely related ecologically and taxonomically to *An. gambiae s.s*, but it is genetically distinct from it. It is genetically more heterogeneous than *An. gambaie s*.s (White, 1981). It is characterized by the eight common inversions including b,c,d, e , on chromosome X , inversion a , on chromosome 3R , and a , b , c , d , e , and f on

chromosome 2R The six 2R inversion systems in *An. arabiensis* are simplified and designated as the 2Ra, 2Rb and 2Rd each with its specific alternative arrangements (Coluzzi *et al,* 1979). A cytogenetic map of the polytene chromosomes indicating the positions of the inversions in the sibling species *An. gambiae* is shown Figure 1.5.



*Mapped only by recombination to 12.8 cM; it is flanked by microsatellites that have been mapped by in situ to 29D (~ 4.7 cM) and 35B (~ 29.1 cM).

Figure 1.5: Cytogenetic map of *Anopheles gambiae* polytene chromosomes indicating the positions paracentric inversions from (Coluzzi et al. 2002).

Analysis of the differences in larval and pupal morphology and genetic variations in populations of *An. arabiensis* in South Africa, has suggested that *An. arabiensis* may be comprised of more than one species (Coetzee, 1997). It is said to be eco – phenotypically elastic and difficult to describe in restricted biological terms. It is found in different ecological conditions and can exploit various niches. Depending on the circumstances and the behaviour of the human host, it can be endophilic, or exophilic, endophagic or exophagic (Coluzzi et al. 1979). *An. arabiensis* is a secondary vector of malaria in most areas of its distribution (Gillies and Coetzee, 1987) (Figure 1.6).

1.5.2 Sibling species and insecticide resistance

A non – uniform reduction of the density of the biting anopheline populations was reported in the villages that were under mosquito control using indoor – house spraying with propoxur (Molineaux *et al*, 1976). Domestic structres in One hundred and sixty villages in Garki district in Nigeria were sprayed with propoxur under a pilot malaria control program. The post control assessement showed variation in the residual anopheline biting density both within and between control and treated villages. This observation was found to correlate later with the predominance of *An. arabiensis* carriers of either the alternative arrangements 2Ra or 2R+a which preferentially rest indoors and outdoors respectively (Coluzzi *et al,* 1979). Recently, analysis of a population of *An. gambiae* complex found the *kdr* allele was associated only with the Savanna form and absent in sympatric populations of Mopti Bamako and *An. arabiensis* (Fanello *et al*, 2003). In the molecular M and S forms of *An. gambiae*, the distribution of the *kdr* allele seems to follow specific patterns in different localities. The *kdr* gene was found at high frequencies among the S – form populations in Burkina Faso and Cote d'Ivoire and was absent from the M at most localities even where it is sympatric with the S form (Chandre *et al,* 1999; Della Torre *et al,* 2001,). However, *kdr* occurred at a frequency of 90% in S and 30% in M forms in Benin (Black and Lanzaro, 2001).

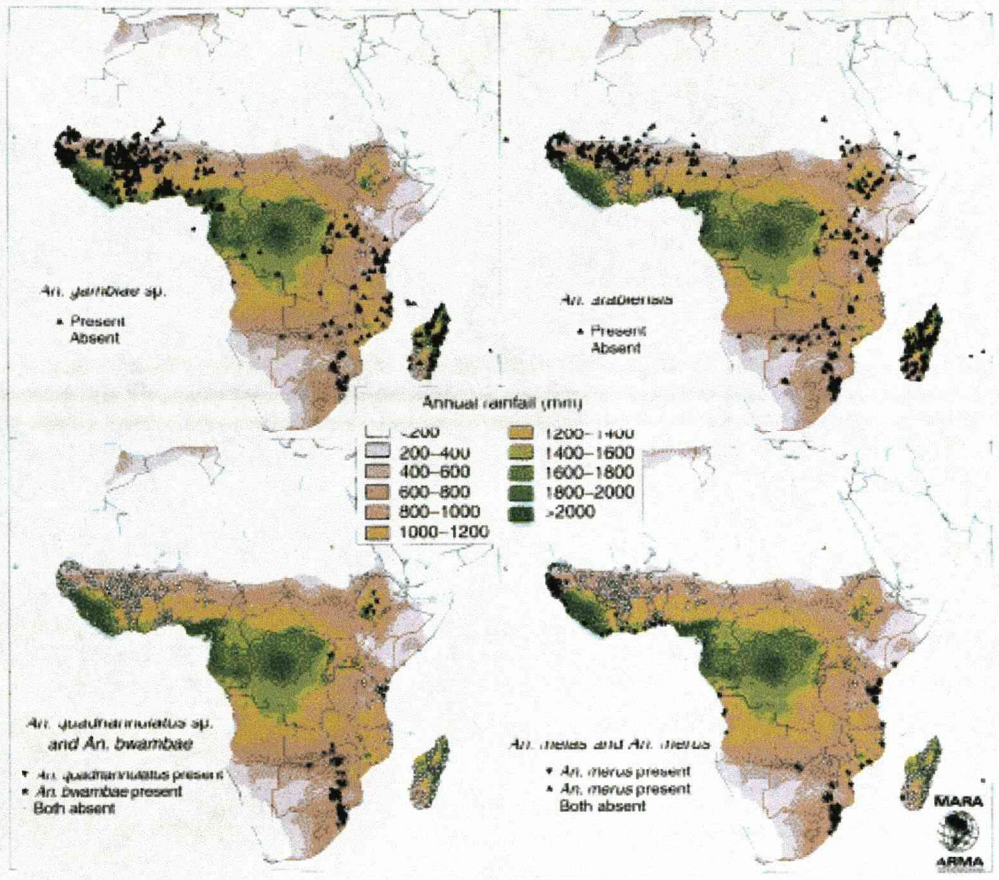


Figure 1.6 Collection sites where *An. arabiensis* and other named species of *An. gambiae* complex have been found (closed symbols), against mean annual rainfall. Open circles indicate collection sites where species were not recorded, although this does mean that they are absent (from Coetzee et al. 2000).

1.5.3 Insecticide resistance and *An. arabiensis*

Resistance to dieldrin among strains of species B of *An. gambiae* complex, later named *An. arabiensis* was first reported in 1955 (Davidson and Hamon 1962). DDT resistance was detected in some field populations of *An. arabiensis* from Sudan and a colony of the strain was selected for DDT resistance in a laboratory in London (Haridi, 1970). Adult *An. arabiensis* in Sudan were resistant to DDT, dieldrin, malathion, phenthoate and permethrin insecticides (Hemingway, 1983). Cross – resistance between malathion and phenthoate and results of biochemical tests with the synergist triphenyl phosphate (TTP) suggested involvement of a malathione – specific carboxylesterase in resistance.

A single partially dominant gene, or group of closely linked genes, has been implicated in control of malathion resistance in Sudanese *An. arabiensis* (Lines *et al,* 1984). Recently, biochemical evidence has suggested partial involvement of GSTs in DDT – resistance in *An. arabiensis* which were collected from an area under malaria control in South Africa (Hargreaves *et al*, 2003). In contrast no resistance to DDT was detected in populations of *An. arabiensis* from 17 localities in Mozambique. However, there is some evidence for low level of resistance to pyrethroids in *An. arabiensis* from five sites at southern Mozambique but no cross resistance to DDT suggesting that this resistance is not caused by the *kdr* mutation (Casimiro *et al,* 2006). A laboratory colony (MBN) of *An. arabiensis* from South Africa was selected for resistance to DDT but lack the *kdr* mutation when assayed. However, the West African L1014F *kdr* mutation was detected in 8 individuals, in the SENN colony from Sudan but the presence of the mutation did not assort with survival to DDT exposure (Matambo *et al*., 2007).

1.6 Objectives of the study

Most of the previous studies on the mechanisms of insecticide resistance in African malaria vectors have focussed on *An. gambiae* s.s. The main objective of this study was to understand the molecular basis of resistance to DDT in *An. arabiensis*. Specifically, bioassay and biochemical assays were conducted to determine the level of resistance and predict the resistance mechanism. The particular role of the Epsilon class of GSTs in DDT resistance was then studied in more detailed. The developmental expression profile, and effect of various xenobiotic inducers on individual GST gene expression was determined. The putative transcription factors that may be involved in regulating the expression of the GSTs were identified.

CHAPTER 2.

LABORATORY SELECTION FOR AND BIOCHEMICAL CHARACTERISATION OF DDT RESISTANCE IN *AN. ARABIENSIS*.

2.1 Introduction

Anopheles arabiensis is the second most efficient malaria vector species of the *An. gambiae* complex and it occurs in sympatry with *An. gambiae* s.s in most areas, the two species forming the most efficient malaria vectorial system in Africa (Powell *et al,* 1999). *An. arabiensis* is the only member of the *An. gambiae* complex found in the rice irrigation areas in Tanzania and Central Kenya (Ijumba and Lindsay 2001, Kamau and Vulule 2006). Its seasonal abundance together with *An. funestus*, which peaks following the onset of rain, is largely responsible for malaria transmission in southern Africa (Coetzee *et al*, 2000). The variable behaviour of *An. arabiensis* females makes them less vulnerable to house spraying than *An. gambiae* s.s (Gillies and Coetzee, 1987). However, the malaria vectors, *An. funestus* and *An. arabiensis,* have been controlled effectively by spraying of houses with DDT in South Africa from 1945 – 1995 without any sign of resistance during this period (Curtis, 2002a). The use of DDT for indoor residual spraying (IRS) has resulted not only in reductions in vector population density, but also in a decline in morbidity and mortality due to malaria (Curtis, 2002b). However, previous studies have shown evidence for development of resistance to DDT in 56 species of anophelines including important malaria vectors such as *An. culicifacies*, *An. albimanus*, *An. stephensi*, *An. funestus*, *An. gambiae* and *An. arabiensis* (Brown, 1986; WHO, 1986). In recent times, approximately 3 years after re-introduction of DDT to control pyrethroid resistant *An. funestus*, resistance to DDT has been reported in field populations of *An. arabiensis* in South Africa (Hargreaves *et al*, 2003). Most earlier studies of insecticide resistance in anophelines have dealt with populations selected in the laboratory for many generations. Hemingway (1981) has investigated in detail the mechanisms of resistance to DDT in eight populations of anopheline mosquitoes including *An. arabiensis* that have been selected to homozygosity for DDT resistance. The MBN (South Africa) and SENN (Sudan) colonies of *An. arabiensis* strains have been selected continuously on increasing doses of DDT over 40

generations until the adults could survive exposure to 4% DDT for more than 1 hour (Matambo et al.2007).

DDT resistance in mosquitoes commonly is due to one of two mechanisms; increasing DDT dehydrochlorination catalysed by GSTs or decreased target – site insensitivity (Hemingway and Ranson, 2000). The latter mechanism confers cross – resistance to pyrethroids. Increased levels of GST activity have been implicated in DDT resistance in *An. subpictus* (Hemingway, et al. 1991), *An. gambiae* (Prapanthadara, et al. 1993) and *An. arabiensis* laboratory colonies (Matambo *et al,* 2007).Using WHO discriminating dosage adult bioassay with permethrin, Hemingway (1983), demonstrated evidence of pyrethroid resistance in the G1 colony of *An. arabiensis* suggesting involvement of *kdr.* Recently, the West Afriacan L1014F mutation has been detected in 8 individuals in the SENN colony from Sudan but this mutation was not detected in the MBN colony from South Africa (Coetzee *et al,* 2006). The objective of this chapter was to select the MAT and KGB laboratory – reared strains of *An. arabiensis* for DDT resistance. A selection regime was established and the LT50 and LT90 values were obtained using dosage/time mortality relationships. Mosquitoes surviving LT50 exposures were sustained into the following generation. Susceptibility data were obtained with WHO test methods (WHO, 1998). Samples of mosquitoes from the parental and selected lines for both strains were subjected to biochemical analysis and assayed for possible *kdr* mutations.

2.2 Materials and Methods

2.2.1 Colonisation of *An. arabiensis*

2.2.1.1 Mosquito Strains

Eggs of *An. arabiensis* MAT strains were collected from a field site at Matatuine, 10 Km from Maputo in Mozambique. The colony was first established at the Institute Nacional de Saude Mozambique in May 2000 and transferred to the Liverpool School of Tropical Medicine in 2002. No information was available on the resistance status of this colony to any class of insecticide. Adult females of *An. arabiensis* KGB stains were caught at Kayemba, Zambesi Valley in Zimbabwe. A colony was first established at the Blair Research Institute in Zimbabwe in 1975 and subsequently at

the South African Institute for Medical Research. Eggs were brought on request to Liverpool by Dr Basil Brooke in September 2004 and a colony was re-established there. Although this strain was said to be susceptible to DDT (Brooke, Personal Communication), a low level of resistance was detected in the F1 generation and hence the strain was maintained on selection with 4% DDT.

2.2.1.2 Mosquito Rearing

Parental and selected lines of *An. arabiensis* MAT and *An. arabiensis* KGB strains were maintained in the insectaries for the four year duration of this study, to provide a range of biological material for the biochemical and molecular experimental work that was undertaken.

2.2.1.3 Rearing Procedure

All colonies were maintained in the insectaries at the Liverpool School of Tropical Medicine. The temperature and relative humidities were recorded using a Mason's wet and dry bulb thermometer. Temperatures were regulated by a thermostat and generally fluctuated from 27 – 28°C; a humidifier maintained relative humidities of 53 – 85%, depending on the insectary. A 12-hourly day and night cycle was maintained by setting a time switch to the light circuit. All mosquitoes were reared in plastic bowls that were 706.5cm^2 with a depth of 15cm or deep trays of 30cm x 25cm with a depth of 13cm. Emerging mosquitoes were collected using a mouth aspirator from underneath nets that covered these containers. Alternatively, pupae were picked by pipettes into a pupal pot and then placed in the cage to allow the mosquitoes to emerge directly into their new environment. The latter procedure minimises the escape of mosquitoes into the insectary. *An. arabiensis* MAT and *An. arabiensis* KGB strains were all reared in distilled water, but on some occasions when high larval mortalities was observed, normal Liverpool tap water was substituted for rearing the strains. However, at later stages, both *An. arabiensis* MAT and KGB strains were found to do better in distilled water as the larvae were more active compared to those reared in tapwater, and the mortality amongst them was lower. The duration of rearing from eggs to emerging adults ranged from 7-12 days amongst all the strains. All mosquito larvae were fed on Tetramin fish food flakes, using ground-up flakes for the first instar. Sufficient food for the larvae was added whilst preventing a film forming on the water surface. A small amount of distilled water was added into the larval trays on

a daily basis to aerate the water. The water was only changed if it became cloudy or the swimming behaviour of the larvae was observed to be sluggish. Extreme care was taken to avoid contamination between the different lines and strains of *An. arabiensis.* All larval trays were cleaned with hot water after each rearing cycle, when all the pupae had emerged into adults and before setting up a new batch of eggs. After drying the trays were swabbed with cotton wool moistened with 70% ethanol. Pipettes, egg pots and larval trays were colour-coded for each strain of *An. arabiensis* strains. Adult mosquitoes were constantly provided with cotton wool soaked in saturated 10% sugar solution in tap water. Initially, females of the parental line *An. arabiensis* MAT were given guinea pig blood twice a week. This was done by placing an anaesthetised guinea pig over the top of the cage, usually between 11.30 and 13.00 hours, and allowing the mosquitoes to feed through the netting of the cage. Later, the source of blood was shifted to human.

2.2.2 Susceptibility WHO bioassays

2.2.2.1 Testing mosquitos from *An. arabiensis* MAT and KGB stains for susceptibility to DDT

Adult mosquitoes from the colonies of the parental lines of *An. arabiensis* MAT and *An. arabiensis* KGB strains were tested for susceptibility to DDT. Bioassays were performed according to WHO protocols using standard WHO susceptibility test kits and 4% DDT impregnated papers. Batches of 20 to 25 non-blood fed adult mosquitoes were tested per assay and no more than six assays were performed for each paper. In most cases one-day old adults of both sexes were used in the assay. Prior to exposure, mosquitoes were transferred to holding tubes (green-dot tube) for one hour, after which damaged or moribund specimens were excluded. The mosquitoes were then gently blown into the exposure tube via the connecting slide. The slide was then closed and the holding tube detached and placed on the table in the horizontal position (WHO, 1998). The times for the exposure to DDT were either 15, 30, 45 or 60 minutes. After exposure for each time point, mosquitoes were maintained in the holding tubes for 24 hours before mortality was scored. Each test was replicated at least four times with different mosquito batches, to take into account inter-batch variability. Survivors from each test were placed in a separate cage and used to establish subsequent generations. To ensure that mortality was attributable to

the insecticide exposure, mosquitoes were exposed to untreated paper impregnated with carrier oil as a control in each test. Subsequently, bioassay tests were repeated with samples of mosquitoes drawn from the selected lines of the two *An. arabiensis* strains. Batches of mosquitoes from *An. arabiensis* MAT and KGB selected lines were tested for resistance to DDT and cross-resistance to permethrin. In the subsequent susceptibility tests, samples of adult mosquitoes from F10 and F20 selected generations of *An. arabiensis* MAT and *An. arabiensis* KGB respectively were exposed to 4% DDT and 0.75% permethrin for one hour. The number of knocked-down mosquitoes was recorded at 10, 20, 30, 40, 50 and 60 minutes. The KDT50 and KDT90 knock-down times were calculated by probit analysis. The data were entered into Minitab 14 and LDP line software programmes for analysis.

2.2.2.2 Selection for resistance to DDT

Mosquitoes from the *An. arabiensis* MAT and KGB strains, which survived previous exposures to DDT, were reared and their progeny subjected to selection using 4% DDT. Adult mosquitoes from *An. arabiensis* MAT strain were maintained under selection pressure with 4% DDT continuously from April to November 2003 and after an interruption, selection was continued from May to December 2004.

Cohorts of 10 to 25 adult mosquitoes of both sexes were placed in WHO exposure tubes, with many replicates per generation (depending on the availability of mosquitoes), and exposed to DDT for 30 minutes. Exposure times were increased to 40, 45, 50 and 60 minutes gradually as the mortality decreased through selected generations. Final mortality for each cohort was recorded 24-hour post-exposure. Survivors from each test were put back into a new cage, blood fed and reared for further selection. Mosquitoes from the *An. arabiensis* KGB strain were selected similarly for resistance to DDT in November 2004, from February to December 2005 and March to November 2006.

2.2.3 PCR for species identification and *kdr*

2.2.3.1 Extraction of genomic DNA

Genomic DNA was extracted using the LIVAK buffer extraction method described by Collins *et al.,* (1987). The DNA was extracted from a batch of four individual one-day old adult mosquitoes from a colony of *An. arabiensis* MAT strain parental line or *An. gambiae* Kisumu or ZAN/U strains. Each batch was homogenised in 100µl of LIVAK buffer (0.13M Tris-HCl-containing 80mM NaCl, 0.16M sucrose, 0.05M EDTA, 0.5% (W/V) SDS, pH 8.0) and the solution was incubated at 65°C for 30 minutes. Potassium acetate (8M) was added to a final concentration of 1 M and the homogenate was mixed and incubated on ice for 10 minutes. The sample was centrifuged at 12000g, 4°C for 20 minutes and the supernatant was transferred to a new tube. After precipitating DNA with cold absolute ethanol and washing twice with 70% (V/V) ethanol to remove the salt, the pellet was re-suspended in 100µl TE (10mM Tris-HCl, 1mM EDTA, pH 8.0) and the genomic DNA stored at 4°C

2.2.3.2 Species identification by PCR

Genomic DNA extracted from a male and female mosquito randomly picked from each of the *An. arabiensis* MAT, *An. gambiae* s.s. Kisumu and the ZAN/U colonies was used as a template in a PCR reaction, using the species-specific oligonucleotide primers – GA[5' - CTG GTT TGG TCG GCA CGT TT - 3'] and AR [5' - AAG TGT CCT TCT CCA TCC TA - 3'] in combination with a universal primer UN[5' - GTG TGC CCC TTC CTC GAT GT - 3'] (Scott *et al.,* 1993). The primers GA and AR amplify products diagnostic for *An. gambiae* s.s. (390bp) and *An. arabiensis* (315bp) respectively. An optimised PCR reaction mix (25µl) was prepared containing the following: 0.2mM dNTPs, 0.2mM $MgCl_2$, 0.0037mM AR, 0.0012mM GA, 0.0025mM UN, 1x manufacturer's reaction buffer (Qiagen), 0.875 units of Taq polymerase (hotstart) and 1µl DNA of the respective template. The master mix was prepared on ice. The PCR machine was programmed to run at an initial 94°C for 15 minutes to activate the enzyme, followed by 30 cycles of 94°C for 30 seconds (denaturation), 50°C for 30 seconds (annealing), 72°C for 30 seconds (extension) with a final extension step at 72°C for 10 minutes. The PCR products were separated on

2% agarose gels. The species identification was performed to confirm the identity of the colony and check for any contamination with *An. gambiae s.s*

2.2.3.3 Knockdown resistance (*Kdr*) diagnostic assay

A PCR assay described by Martinez-Torres *et al,* (1998) was used to test for the presence of the typical *An. gambiae kdr* allele in the parental stock of *An. arabiensis* MAT. One µl of genomic DNA extracted from the individual mosquoties described in 2.2.3.1, was added as a template to a 25µl PCR mixture containing 0.2mM dNTPS, 2.5 mM $MgCl_2$, 4µM of each of primer Agd1 (5'-ATA GAT TCC CCG ACC ATG-3') and Agd2 (5'-AGA CAA GGA TGA TGA ACC-3'), 1 x manufacturer's reaction buffer (Qiagen) and 1.25 units Taq DNA polymerase (hotstart). PCR reaction conditions were 94°C for 15 minutes, 94°C for 1 minute, 48°C for 2 minutes, 72°C for 2 minutes for 40 cycles, 72°C for 10 minutes (Martinez-Torres *et al,* 1998). The amplified fragments were analyzed using a 2% agarose gel stained with ethidium bromide and visualized under UV light. The DNA fragment indicating the common band was subcloned and sequenced.

The PCR was repeated as described above but adding to the reaction mixture 2µM each of the diagnostic primers: Agd3 (5' – AAT TTG CAT TAC TTA CGA CA-3'), and Agd4 (5'- CTG TAG TGA TAG GAA ATT TA-3').

2.2.4 Biochemical analysis

2.2.4.1 Biochemical assays

Mosquito samples taken from the parent stock and F15 selected generations of *An. arabiensis* MAT and KGB strains were kept at -80°C for biochemical analysis. Biochemical assays were performed according to the standardized procedures described in the manual by Hemingway (WHO 1998). Batches of 22 one-day old, frozen mosquitoes were individually homogenized in 200µl of distilled water in 1.5ml eppendorf tubes, each of which was numbered to correspond to a specific specimen. Homogenization was carried out manually on ice using a blue plastic pestle. The crude homogenate was spun at maximum speed for two minutes in a microfuge. After centrifugation, the supernatant from each eppendorf tube was then transferred to a well of a microtitre plate corresponding to the number of the specimen. Esterases,

monooxygenases and GST assays were carried out as described by (Hemingway 1998). The microtitre plates with the homogenates were kept on ice until analysis. Buffer solutions for all the biochemical assays were kept at room temperature, 30 mM σ –napthyl acetate (1-NA) and 30 mM β-napthyl (2-NA) were kept at 4°C. All other solutions were prepared immediately before the asays were carried out.

2.2.4.2 Esterase assays

Non-specific esterase activity was measured with the substrates σ and β naphthyl (NA). Two 20µl replicates of supernatant from each sample were transferred to fresh microtitre plates. To the first replicate, 200 µl of 30mM σ-naphthyl acetate (1-NA) in phosphate buffer 0.02m (PH 7.2) was added and to the second replicate, the same amount of β-naphthyl acetate (2-NA) acetate solution. The enzyme reaction was left for 30 minutes at room temperature. Fifty µl of fast blue stain solution (22.5mg fast blue in 2.25ml in distilled water, plus 5.25ml of 5% sodium lauryl sulphate diluted in 0.1M phosphate buffer (pH 7.0), was added and incubated for 5 minutes to stop the reaction. The concentration of the products from the enzyme reaction was calculated by reading absorbance at 570nm in the thermomax plate reader as an end point. Optical densities for individual mosquitoes were compared with standard curves of optical densities for known concentrations of the products 1-naphthol and 2-naphthol respectively. The results were reported as product formed/min/mg protein.

2.2.4.3 Glutathione S-transferase assay

Two replicates of 10µl aliquots of homogenates from each sample was transferred to new microtitre plate wells. The GST activity was measured by addition of 200 µl of GSH/CDNB working solution (10 mM reduced glutathione prepared in 0.1M phosphate buffer pH 6.5 and 63mM chlorodinitro benzene (CDNB) diluted in methanol) into each replicate. Two blanks were prepared for each plate with 10µl of distilled water and 200µl GSH/CDNB working solution. The rates for the enzyme reaction were measured at 340nM for 5 minutes. The GST activity per individual mosquito was calculated into absolute units based on the extinction coefficient ($9.6mm^{-1}$) and the light path of the solution in the microtitre plate (0.6cm). The result was reported as mMol CDNB conjugated/min/mg protein.

2.2.4.4 Monooxygenase assay

The haem-peroxidase assay, as modified by Brogdon (1997), was used to titrate the total amount of haem containing protein in each mosquito. Eighty µl of 0.0625M potassium phosphate buffer pH 7.2 and 200µl of TMBZ solution (0.01gm of 3, 3', 5, 5' – tetramethyl benzidine in 5ml of absolute methanol mixed with 15ml of 0.25M sodium acetate buffer pH 5.0) were added to 20µl aliquots of mosquito homogenate. 25µl of 3% hydrogenperoxide was added and the mixture was left for two hours at room temperature. Two controls per plate were prepared with 20µl of distilled water, plus the working solution. Samples were read at 650nM and values were compared with a standard curve of known concentration of cytochrome C. The values were reported as equivalent units of cytochrome P450/mg protein corrected for the known haem content of cytrochrome C and P450.

2.2.4.5 Protein assay

Protein assays were based on the method of Bradford (1976). Microfuged homogenate (10µl) from each mosquito was added to 300µl Bio-Rad Protein assay reagent (diluted 5 times from stock), incubated for 5 minutes and end point absorbance measured at 570nM. Protein concentration was determined by converting the absorbance into concentration based on a bovine serum albumin standard curve.

2.3 Results

2.3.1 PCR identification of members An. gambiae complex

A total of 18 individual specimens randomly selected, six each from the colonies of Kisumu and Zan/u strains of *An. gambiae* and the MAT strain of *An. arabiensis* were examined. The PCR for species identification was based on the method of Scott et al.(1993) using specie specific oligo nucleotide primers. The results of the PCR assay indicated that all specimens from the MAT strain were *An. arabiensis* showing the distinctive 315 bp bands. The PCR was conducted to confirm the identity of the colony and check for contamination with *An. gambiae*.

2.3.2 WHO susceptibility assays

The susceptibility levels to DDT of adult mosquitoes of the original parental populations of the *An. arabiensis* MAT and KGB strains were determined. There has

been no previous record of exposure of either of the strains to any class of insecticide and the KGB has been used as a laboratory reference insecticide susceptible strain (Brooke, personal communication). The WHO test kits were used and tests were carried out using the WHO recommended discriminating dosage. A total of 338 two to three day old adult mosquitoes from the parental *An. arabiensis* MAT strains were exposed to 4% DDT for different time periods. The percentage mortality recorded 24 hours after exposure is shown (Table 2.1). The 87% mortality after exposure to DDT for 1 hour is indicating the presence of low level of resistant genotypes in the MAT parental colony.

Table 2.1: Susceptibility of adult mosquitoes in parental MAT colony to DDT

Time (min) Exposure	Number Tested	Number Dead	Number Alive	% Mortality 24 post-exp
15	58	16	42	27.5
30	80	53	27	66.3
45	135	116	19	85.9
60	65	57	8	87.6

Initial scores for mortality from WHO diagnostic test kit for 4% DDT tested against adult mosquitoes (n=338) sampled from the F1 generation of parental line

In the KGB strain, 389 adult mosquitoes in batches of 20 – 25 were exposed at the four different time points and mortality was recorded 24 hours post exposure as shown (Table 2.2). The 81.6% mortality after exposure to DDT for 1 hour suggests higher level of resistance genotypes in KGB than in the MAT colony.

Table 2.2: Susceptibility of adult mosquitoes in parental KGB colony to DDT

Time (min) Exposure	Number Tested	Number Dead	Number Alive	% Mortality 24 post-exp
15	67	13	54	18.6
30	85	38	47	44.7
45	98	65	33	66.3
60	148	121	27	81.6

Initial scores for mortality from WHO diagnostic test kit for 4% DDT tested against adult mosquitoes (n=398) sampled from the F1 generation of parental line

2.3.2.1 Selection of resistant genotypes

After determination of the resistance status of the adult mosquitoes in the colonies of the parental lines of *An. arabiensis* MAT and KGB strains, the colonies were selected for the DDT – resistant genotypes in order to characterise the underlying mechanism. As the susceptibility tests on the MAT parental stock showed, 30 minutes exposure period produced over 66% mortality in the parental population, this dosage was used to select for the resistant genotypes. The selection pressure was increased when a decrease in the percentage mortality was observed over several generations.

DDT selection of *An. arabiensis* MAT strain for 30 minutes produced a drop in mortality after one generation but the mortality kept on fluctuating between the generations one to three.

Table 2.3: Selection of *An. arabiensis* MAT with DDT

Generation	Exp. Time (min)	No. Tested	Dead	Alive	% Mortality
F1	30	143	103	40	72
F2	30	170	109	61	64.1
F3	30	58	41	17	73.5
F4	30	81	54	27	67
F5	30	79	46	33	58.2
F6	30	74	38	36	51.4
F14	30	346	214	132	61.8
F15	30	103	56	47	54.3
F16	30	321	155	166	48.3
F17	45	147	102	45	69.4
F18	45	220	134	86	60.9
F19	45	467	266	201	56.9
F20	45	228	121	107	53.1

The mortality decreased from 73.5% in the F3 to 51.4% in the F6. Due to rearing problems, selective pressure was not applied in generations from F7 to F13. The mortality rose back to 61.8% in F14 but decreased gradually to 48.3% in generation 16. Selection at 45 minutes exposure period raised the mortality to 69. 4% but subsequently decreased to 53% in F20 (Table 2.3) (Figure 2.1).

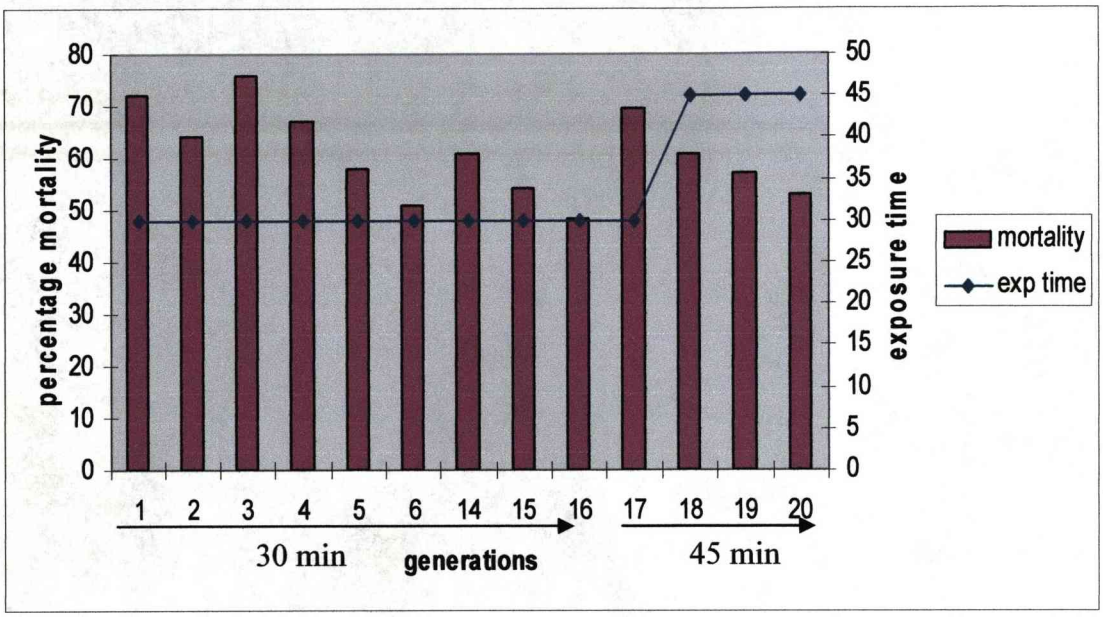


Figure 2.1: Selection of *Anopheles arabiensis* MAT strain with DDT

The KGB colony did show a similar pattern of response to DDT. However, the selection pressure was gradually increased from 30 minutes in F1 generation to 60 minutes over 20 generations. After the initial selection, the colony could not be selected from generations 2 to 5 due to unusually high larval mortality and decreased fecundity in the adult females. Selection pressure was then reapplied continuously at 40 minutes exposure period from generations F6 to F15 except (F9 – F10). Over these selected generations, the mortality decreased from 56.4% in F6 to 28.4% in F14 (Figure 2.2).

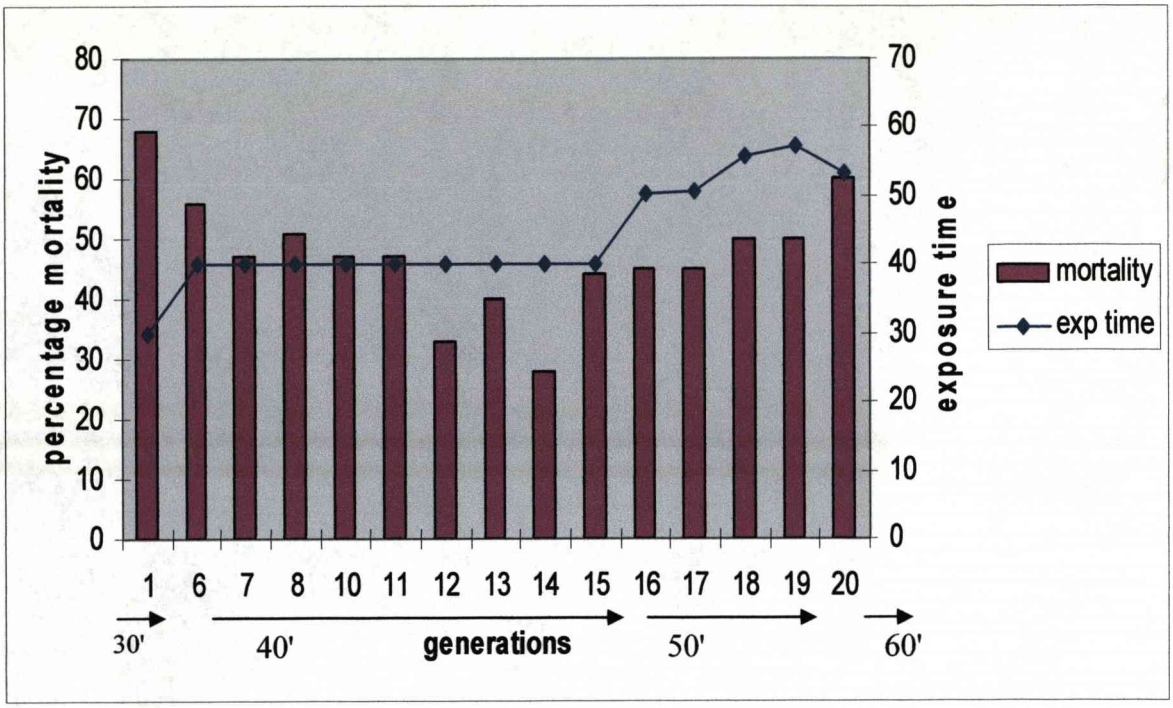


Figure 2.2: Selection of adult *Anopheles arabiensis* KGB strain with DDT

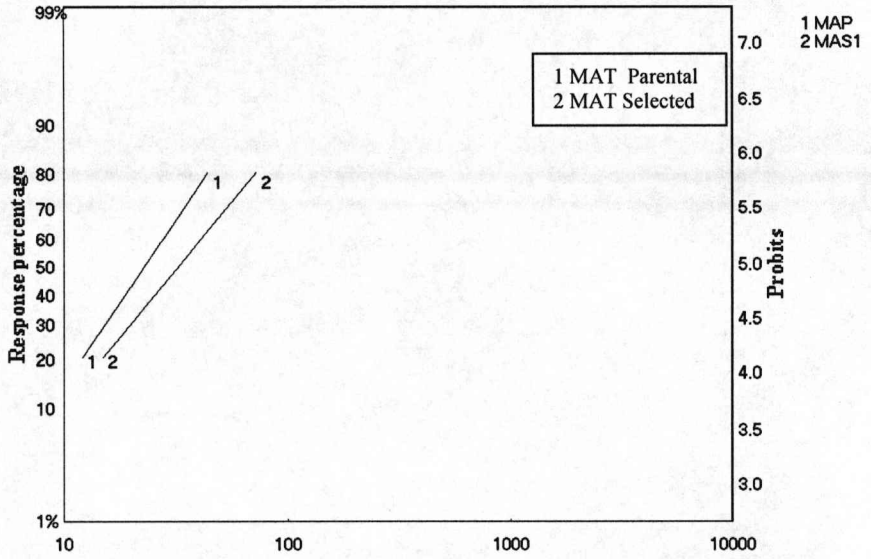
Adults of *An. arabiensis* KGB strain maintained on continuous selection pressure with 4% DDT at 30 minutes in generations F1, F2- F5, no selection due to population bottle neck, selection pressure increased to: 40 (F6 – F13, 50 (F14 –F18) and 60 minutes (F19 – 20) respectively. When the selection pressure was increased to 50 minutes, the mortality increased to 50.3% in F16 and shifted to 55.8% in F18. Generations F19 to F20 were selected at 60 minutes and the mortality increased to 60% (Table 2.4).

Table 2.4: Selection of *An. arabiensis* KGB Strain with DDT

Generation	E. Time (min)	No. Tested	Dead	Alive	% Mortality
F1	30	145	98	47	67.6
F6	40	236	133	103	56.4
F7	40	88	41	47	46.6
F8	40	140	71	69	50.7
F11	40	138	65	73	47.1
F12	40	180	60	120	33.3
F13	40	59	24	35	40.6
F14	40	95	27	68	28.4
F15	50	103	48	55	46.6
F16	50	233	117	116	50.3
F17	50	280	91	189	50.5
F18	50	154	86	65	55.8
F19	60	316	181	135	57.2
F20	60	295	179	116	60.6

After selection, the susceptibility tests with the diagnostic dose of DDT (4%) were repeated at different time points for the parental and selected populations of both *An. arabiensis* MAT and KGB strains. The percentage mortality as recorded and plotted against the exposure time is shown (Figure 2.3)

A



Exposure time (minutes)

B

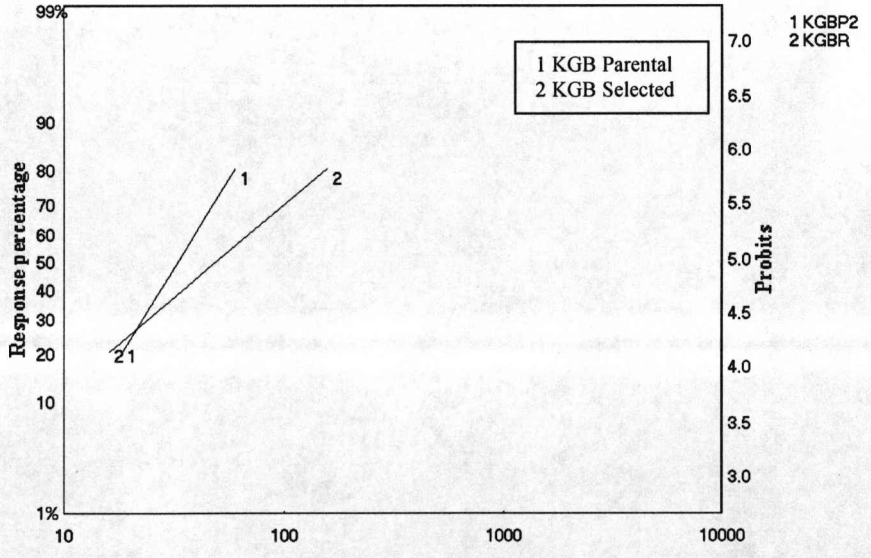


Figure 2.3 B: Time – response curves for the F20 generations of the parental lines of MAT (MAP), and selected lines (MAS) (A) parental (KGBP), and selected lines (KGBR) (B) of the KGB strains exposed to exposed to 4% DDT

The LT50 values for DDT were 23.4 min and 33.2 min (resistant ratio 1.4) in the parental and selected colonies of *An. arabiensis* MAT strain. The slopes of the regression lines are 2.96 in the parental and 2.4 in the selected lines respectively (Table 2.5). In the KGB strain, the LT50 values were 33.5 min and 50.8 min and the corresponding slopes of the regression lines were 3.26 and 1.7 in the parental and populations respectively (Table 2.5). The change in slope of regression lines between KGB selected and parental indicates increased resistance in the selected population.

Table 2.5: Relative susceptibility of DDT (4%) based on time mortality relationships tested against parental and selected lines of *An. arabiensis* MAT and KGB strains

Strain	Line	Sample	No tested	LT50 (min)	CI	LT90	Slope	RR	X^2
MAT	**Parental**	F20	355	23.4	(20.3 – 26.1)	63.40	2.9 ± 0.3	1	0.703
	Selected	F20	290	33.2	(29.2 – 37.7)	88..96	2.40 ± 0.3	1.4	1.301
KGB	**Parental**	F20	360	35.1	(28.2 – 37 .3)	95.06	3.26 ± 0.5	1.5	0.821
	Selected	F20	581	50.8	44.1 – 66.6)	139.56	1.70 ± 0.4	2.2	1.25

The knockdown rates recorded at 10 min intervals of 1 – 3 day old F15 and F18 generations adults from the parental and selected lines of *An. arabiensis* MAT strain is shown (Figure 2.4).

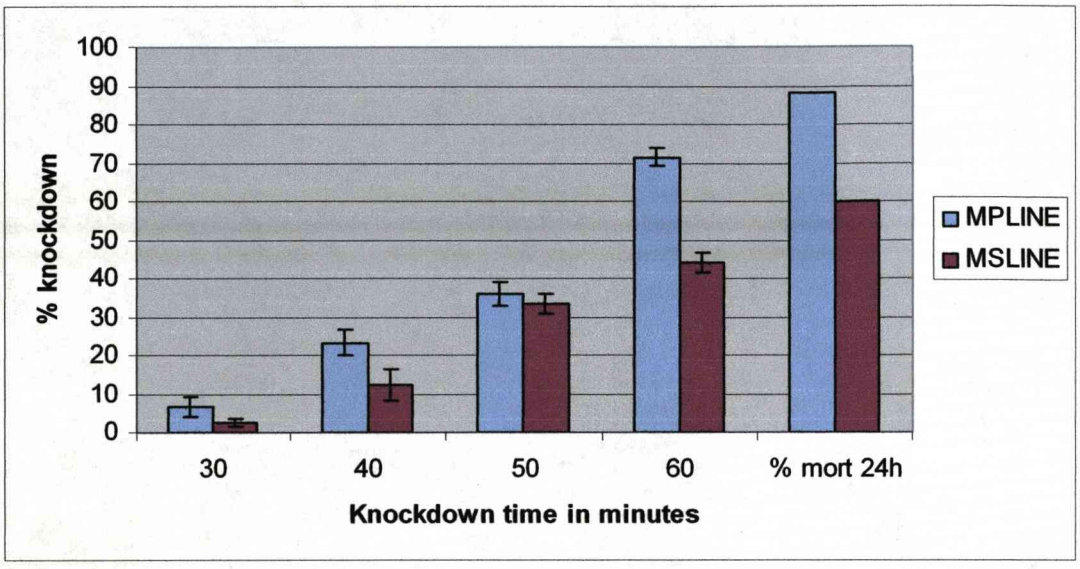


Figure 2.4: percentage knockdown and percentage mortality of 1 – 3 day old adult *An. arabiensis* F15 on 4% DDT.

MPLINE (MAT parental line) (n = 246) and F18 MSLINE (MAT selected line) (n = 207) during 30 – 60 minute exposure to 4% DDT and 24 h after exposure respectively. Fisher's Exact Test statistics was used to calculate P values.

The percentage of mosquitoes knocked down was significantly lower at 30 min ($P = 0.044$), 40 min, ($P = 0.04$) and very significant at 60 min ($P < 0.001$) in the selected than in the parental lines. This indicates a significant increase in resistance to DDT in the selected population.

2.3.2.2 Cross – resistance to permethrin

The populations of *An. arabiensis* DDT selected MAT strain and the parental strain were also tested against the diagnostic dosage of 0.75% permethrin to check for cross resistance or increased tolerance (Figure 2.5). Significantly more mosquitoes were knocked down by permethrin at 30 min, 40 min, ($P < 0.001$) and at 50 min ($P = 0.072$) in the parental than in the DDT selected population but both showed > 97% mortality at 24 hours after exposure (Figure 2.5).

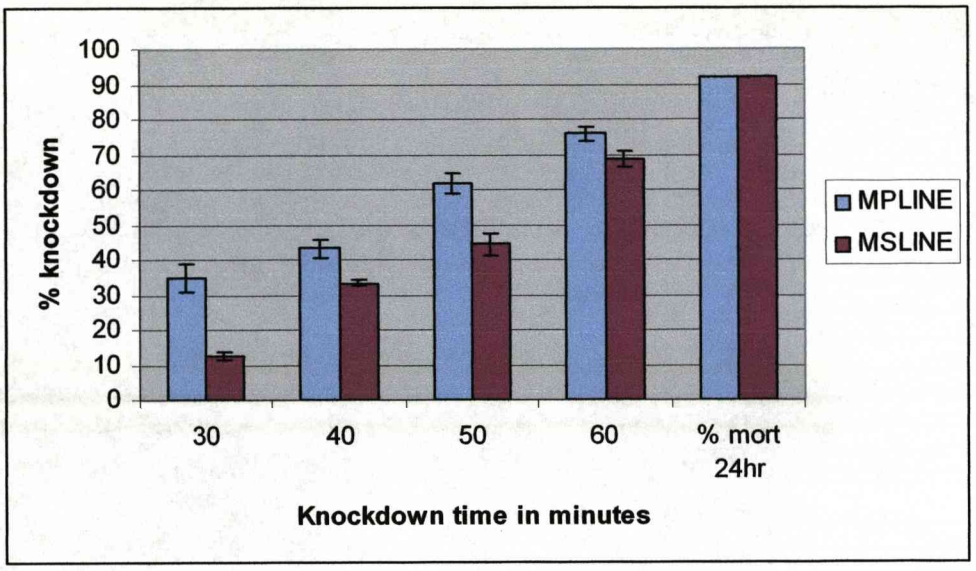


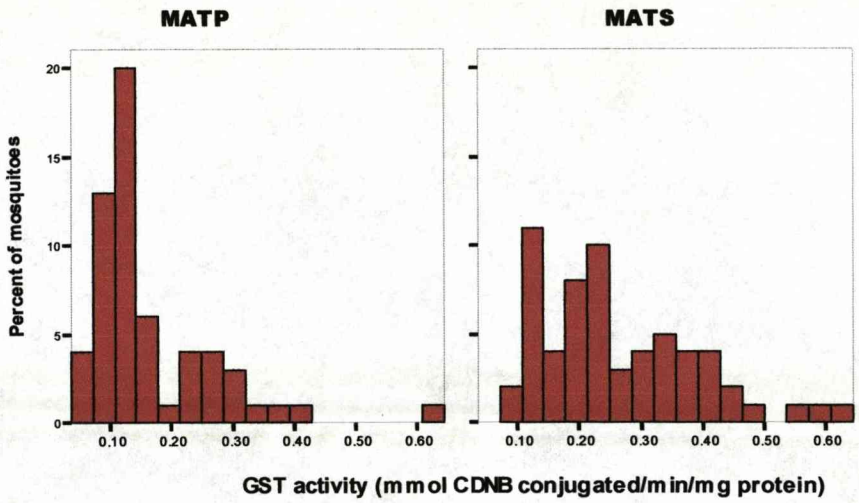
Figure 2.5: percentage knockdown and percentage mortality of 1 – 3 day-old adult F18 on 0.75% permethrin.

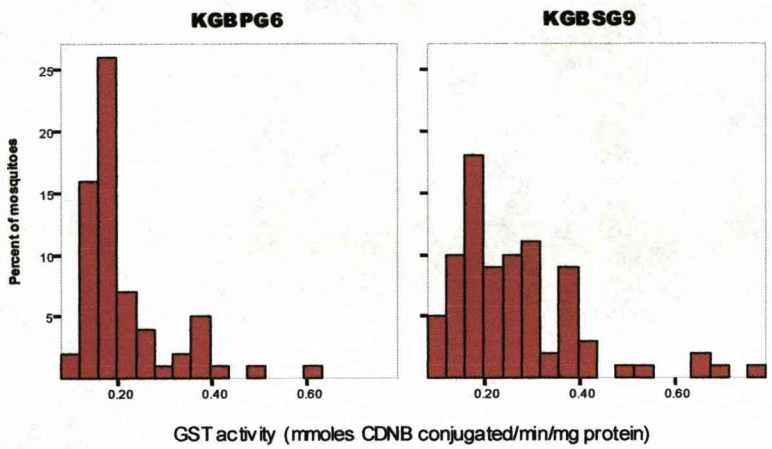
MPLINE (MAT parental line) (n = 261) and F18 MSLINE (MAT selected line) (n = 144) during 30 – 60 minute exposure to 0 .75% permethrin and 24 h after exposure respectively.

2.3.3 Biochemical assays

2.3.3.1 Glutathione S – transferase activity

Elevated GST was detected by assaying the total GST activity of the individual mosquito with the general GST substrate chlorodinitrobenzene (CDNB). Frequency distributions for the GST – activity of one – day old mosquitoes from the parental and DDT selected populations of both the MAT and KGB strains are shown in Figure 2.6. The average GST activity in the *An. arabiensis* Durban insecticide susceptible strain was 0.31 mmol CDNB conjugated/min/mg protein, indicating the normal activity value in a susceptible population (Casimiro *et al*, 2006). However, higher number of mosquitoes in the selected compared to the parental lines of MAT and KGB strains had GST activity higher than the Durban strain (Figure 2.6). This indicates that more DDT resistant individuals with high GST activity are segregating in the MAT selected populations.





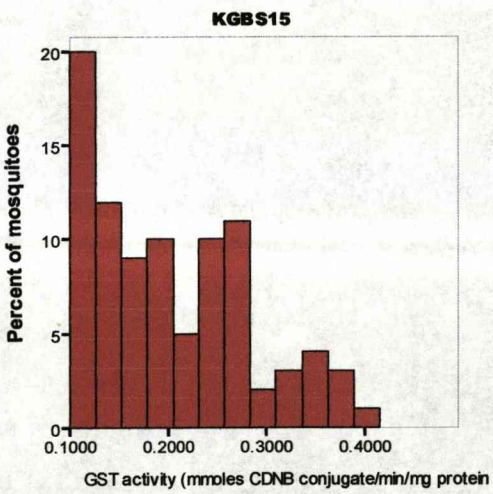


Figure 2.6: Distribution pattern of glutathione S – transferase activity in:MAT parental (n = 59), MAT selected (n = 61), KGBPG6 (n = 60), KGBS G9 (n = 70), KGBS G15 (n = 92). N.B : scales are different on x – axis.

The GST activities followed a log – normal distribution and comparisons were made using geometric means with their 95% confidence intervals. The geometric mean GST activity was significantly higher $p < 0.001$, 0.014 in populations under selection pressure than in the parental (unselected) populations in *An. arabiensis* MAT and the nineth generation KGB strains respectively (Table 2.6).

Table 2.6: Comparisons of geometric means (with 95% confidence limits) for GST activity in *An. arabiensis* MAT and KGB strains

Variable	Line	n	Mean (95% confidence interval)		Test statistic	p-value
GSTact	Mparental	59	0.139	(0.120 – 0.161)		
	Mselected	61	0.236	(0.209 – 0.267)	Mp vs Ms	< 0.001
	KGBPG6	60	0.183	(0.170 – 0.198)		-
	KGBSG9	70	0.221	(0.202 – 0.241)	Kgbg6 vs g9	0.014
	KGBSG15	92	0.189	(0.173 – 0.205)	Kgbg6 vs g15	0.022

Statistical analysis of GST activity (mmol CDNB conjugated /min/mg protein) in the samples from the parental and selected lines of *An. arabiensis* MAT and KGB strains.
Test statistics: GSTact:- $F(118) = 5.579$ MAT $p < 0.001$, $F(2,219) = 5.002$ KGB $p = 0.014$. KGBPG6 denotes KGB parental generation 6, KGBSG9, KGB selected generation 9, KGBSG15, KGB selected generation 15.

In the KGB strain the geometric mean activity was significantly higher $p = 0.014$ and $p = 0.022$ in selected generation (G9) than in the parental (G6) and selected (G15) respectively (Table 2.6). Although the GST activity was also higher in the selected (G15) than in the parental (G6), the difference was not significant. This suggests that GST activity in the KGB strain does not correlate accurately with the level of DDT resistance. The mean GST activities ranged from 0.209 – 0.267 µmol /min /mg and 0.202 – 0.241 µmol /min/mg for the selected lines of MAT and KGB strains respectively. Susceptible strains of *An. arabiensis* from Mozambique and South Africa had GST activities of 0.117 and 0.225 respectively (Casimiro *et al*, 2006; Hargreaves *et al*, 2003). From Figure 2.6, it is clear that a small number of

individuals in the selected lines of both MAT and KGB strains have elevated GST activity (Figure 2.6).

2.3.3.2 Esterase activity

Individual esterase activities (using α – naphthol and β – naphthol as substrates) in the selected and unselected (parental) populations of *An. arabiensis* MAT strain are shown in Figures 2.7 and 2.8

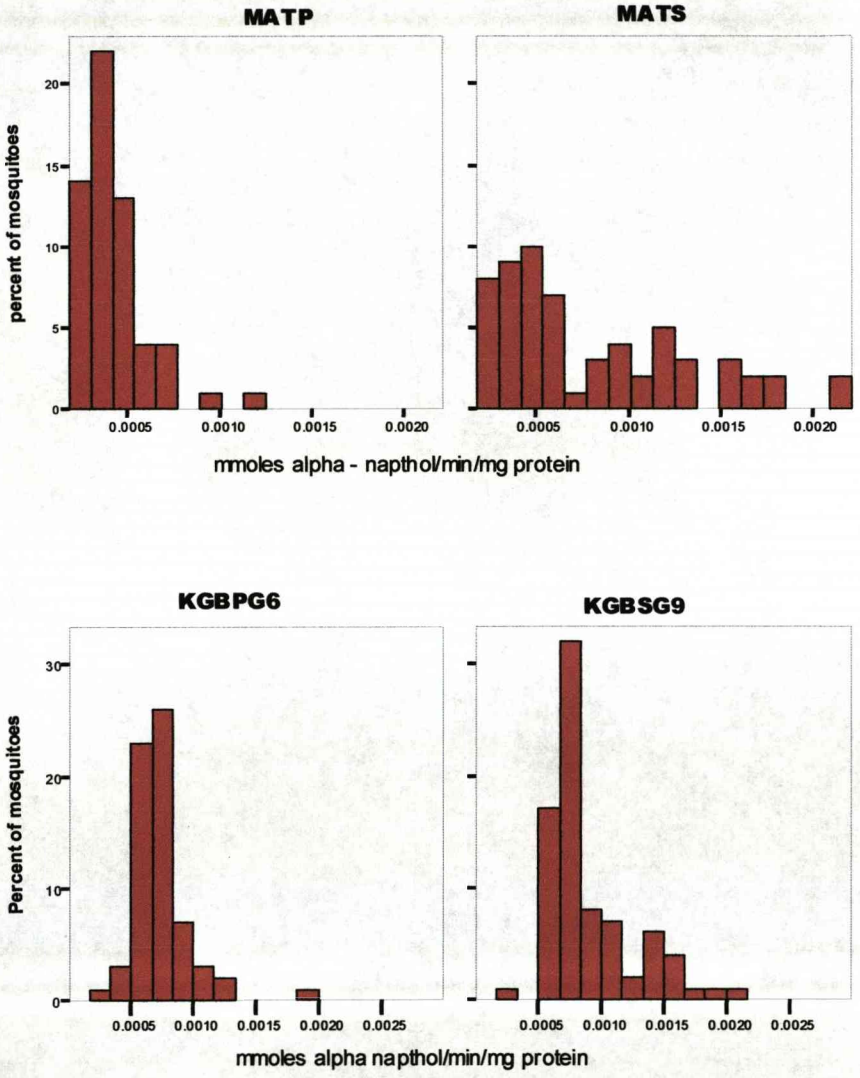


Figure 2.7: Esterase alpha activity profiles in samples from the MAT parental (59) and selected (61) and KGB parental G6 (60) and selected G9 (69) *An. arabiensis* strains. N.B : scales on the y – axis are different.

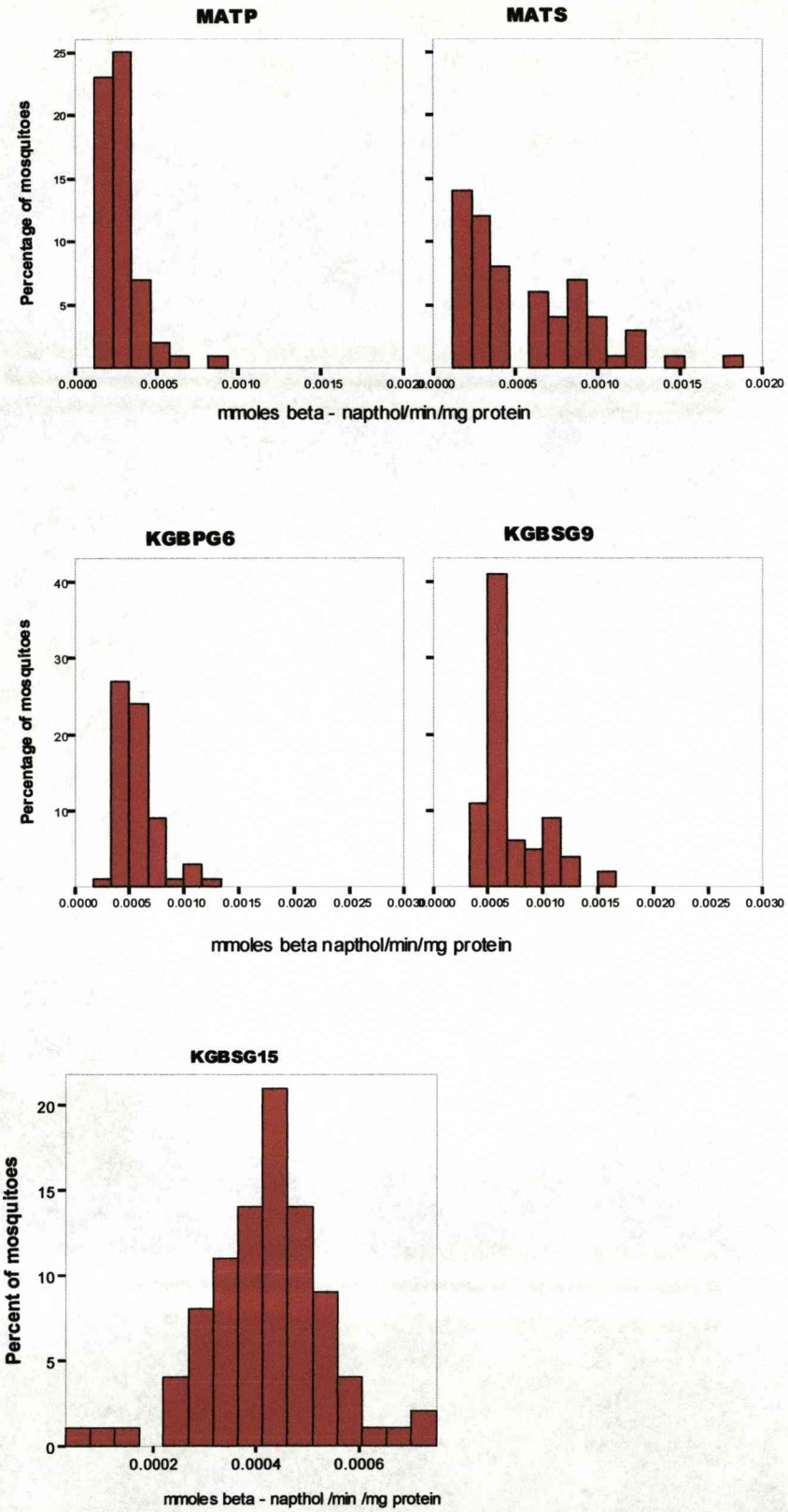


Figure 2.8: Esterase beta activity profiles in samples from the MAT parental (59) and selected (61) and KGB parental G6 (60) and selected G9 (69), G15 (92) *An. arabiensis* strains. N.B scales on the x and y – axis are different.

In the MAT strain, the distribution pattern showed higher esterase activities with each of the substrates in the selected compared to parental populations. However, in the KGB strain, although the esterase activities are higher in the KGBS9 compared to KGBP6, the activities in KGBSG15 were lower than that in KGPG6.The geometric mean values of esterase activities with the two substrates were significantly higher $p < 0.001$ in these cases in the selected than in the parental lines in the strains (Table 2.7). The highest values were observed for KGB selected (G9), these values are 8 – fold higher than that reported for susceptible *An. arabiensis* field strains from Mozambique, but they are still below the level seen in susceptible Durban strain (Casimiro *et al,* 2006).

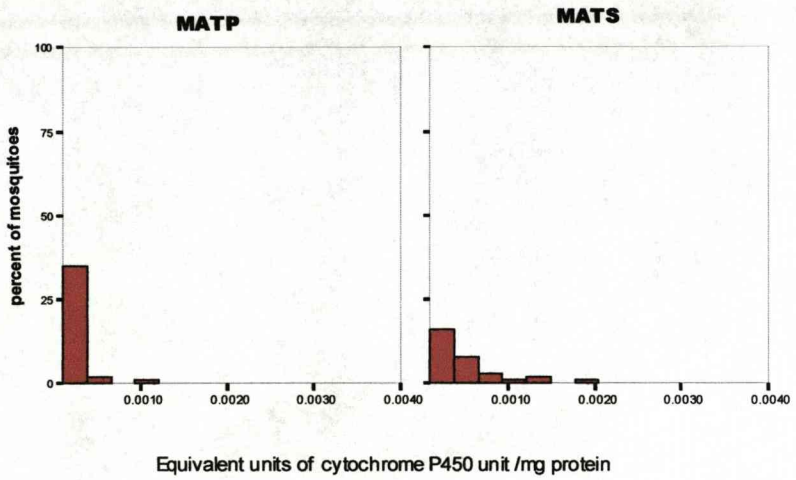
Table 2.7: Comparisons of geometric means (with confidence limits) for a range of biochemical assays between adults sampled from the parental and selected lines of *An. arabiensis* KGB strain

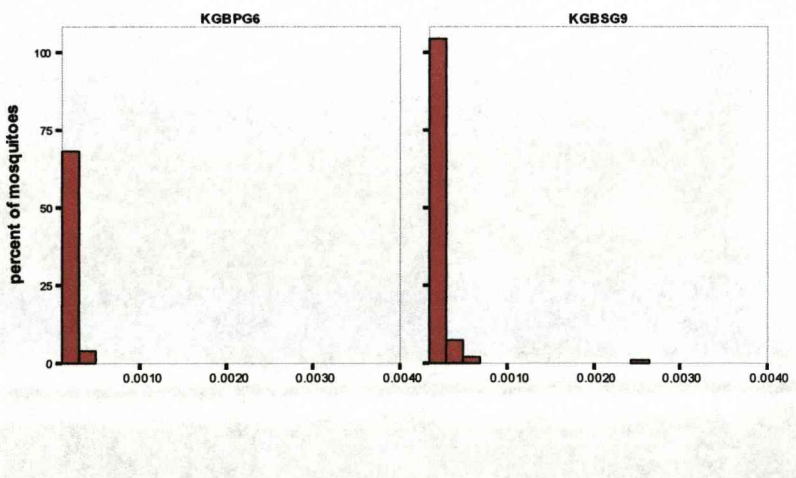
Variable	Line	n	Mean (95% confidence interval)		Test statistic	p-value
Alfact	Mparental	59	0.000399	(0.000363 – 0.000438)		
	Mselected	61	0.000653	(0.000551 – 0.000774)	Mp vs Ms	< 0.001
	KGBPG6	60	0.000700	(0.000655 – 0.000749)		-
	KGBSG9	69	0.000862	(0.000791 – 0.000940)	Kgbg6 vs g9	0.004
	KGBSG15	107	0.000583	(0.000539 – 0.000630)	Kgbg6 vs g15	<0.001
Betact	Mparental	59	0.000271	(0.000247 – 0.000297)		
	Mselected	61	0.000447	(0.000373 – 000535)	Mp vs Ms	<0.001
	KGBPG6	60	0.000536	(0.000504 – 0.000571)		
	KGBSG9	70	0.000670	(0.000615 – 0.000729)	Kgbg6 vs g9	< 0.001
	KGBSG15	92	0.000423	(0.000392 – 0.000456)	Kgbg6 vs g15	<0.001

Test statistics: Alfact: – $F(118) = 5.017$ (MAT), $F(2,235) = 24.857$ (KGB), $p < 0.001$
Betact:- $F(118) = 4.891$ (MAT), $F(2,235) = 36.685$ (KGB), $p < 0.001$

2.3.3.3 Monooxygenase activity

The MAT and KGB *An. arabiensis* strains were homogeneous for low monooxygenases content, as estimated from bound haem titration levels. There were small number of individuals from the selected MAT and KGB (G9) and (G15) with elevated levels of monooxygenases (Figure 2.9).





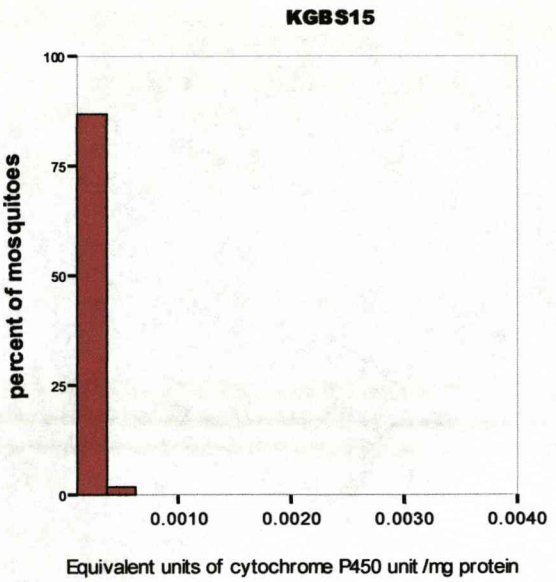


Figure 2.9: Estimated levels of cytochrome P450 (representing monooxygenase activity) in *An. arabiensis* parental MATP (38), selected MATS (32) and parental KGBPG6 (72) and selected KGBSG9 (114), KGBSG15 (89). The numbers in bracket indicate the sample sizes.

The geometric mean values for the monooxygenase activity in the different lines in the two *An. arabiensis* strains is given (Table 2.8). The geometric means for KGBPG6, KGBSG9, KGB G15 and MAT parental lines did not differ significantly ($p = 0.148$ to $p = 0.667$).The monooxygenase activity in these four lines are much higher than in laboratory susceptible *An. arabiensis* Durban strain (Casimiro *et.al.* 2006). The mean level for the MAT selected line was significantly higher than for all four other lines ($p < 0.001$ in all cases). However, only 32 specimens were analysed due to technical problems, a larger sample size may provide a more accurate estimate of the mean enzyme activity in that population. There are several reports linking elevated levels of P450s in *An. gambiae* to insecticide metabolism in particular pyrethroid metabolism (Vulule *et al*, 1999, Ranson *et al*, 2002). Elevated levels of P450 in *An. funestus* in southern Mozambique confer resistance to pyrethroids (Brooke *et al*, 2001, Casimiro *et al*, 2006).

Table 2.8: (Geometric) mean monooxygenase activity levels (with 95% confidence intervals) in *An. arabiensis* MAT and KGB strains

Strain	Line	n	Mean (95% confidence interval)		Test statistic	p-value
MAT	Parental	38	0.000192	(0.000163 – 000227)	F(4,340)= 27.5	-
	Selected	32	0.000410	(0.000300 – 0.000559)	"	0.001
KGB	KGBGP6	72	0.000171	(0.000158 – 0.000184)	"	-
	KGBSG9	114	0.000186	(0.000173 – 0.000201)	"	0.148
	KGBSG15	89	0.000161	(0.000150 – 0.000172)	"	<0.677

Although the mosquitoes from all the KGB lines were not tested for pyrethroid resistance, the MAT selected line has shown increased tolerance to permethrin compared to the parental, but it is not clear if elevated monooxygenases are involved.

2.3.3.4 Detection of knockdown resistance allele (*Kdr)* by diagnostic PCR

The *kdr* diagnostic assay produces three bands (of sizes 137 bp, 195 bp,and 293 bp) for individual mosquitoes that are heterozygous, two bands each for either homozygous resistant (195 bp and 293 bp) and homozygous susceptible (137 bp and 293 bp). In this study, genomic DNA extracted from 10 individual specimens was used as template in the PCR to amplify the 293 bp fragment using Agd1 and Agd2 primers in *An. arabiensis* MAT strain. The PCR products were cloned and sequenced. The positions of triplets encoding leucine in susceptible individuals were identified and compared to *An. gambiae* (Figure 2.10). Indistinct bands were produced in repeatitive multiplex PCR experiments using the diagnostic Agd3 and Agd4 primers and these were not sequenced.

```
                    10         20         30         40         50
>Anarabkdr  ---------- -------CCC CGACCATGAT CTGCCAAGAT GGAATTTTAC
>Angambiae  GATAATGTGG ATAGATTCCC CGACCATGAT CTGCCAAGAT GGAATTTTAC

                    60         70         80         90        100
>Anarabkdr  AGATTTCATG CATT-CCTTC ATGA-TTGTG -TTCCGTGTG CTATGCGGAG
>Angambiae  AGATTTCATG CATT-CCTTC ATGA-TTGTG -TTCCGTGTG CTATGCGGAG

                   110        120        130        140        150
>Anarabkdr  -AATGG-AT- TGAAT-CAAT G-TGGGAT-T GTATGCTTGT CGGTGATGTA
>Angambiae  -AATGG-AT- TGAAT-CAAT G-TGGGAT-T GTATGCTTGT CGGTGATGTA

                   160        170        180        190        200
>Anarabkdr  TCCTGCATAC CATTTTTCTT GGCCACTGTA GTGAT-AGGA AATTTAGTCG
>Angambiae  TCCTGCATAC CATTTTTCTT GGCCACTGTA GTGAT-AGGA AATTTAGTCG

                   210        220        230        240        250
>Anarabkdr  TAAGTAATGC AAATTA---A CATGGAC-CA AGATCGTTTT TACATGA--C
>Angambiae  TAAGTAATGC AAATTA---A CATGGAC-CA AGATCGTTTT TACATGA--C

                   260        270        280        290        300
>Anarabkdr  ATTGTTTTGC A-GGTGCTT- -AACCTTTTC TTA------- GCCTTGCTTT
>Angambiae  ATTGTTTTGC A-GGTGCTT- -AACCTTTTC TTA------- GCCTTGCTTT

                   310        320        330        340        350
>Anarabkdr  TGTCAAATTT TGGTTCATCA --TCCTTGTC T--------- ----------
>Angambiae  TGTCAAATTT TGGTTCATCA --TCCTTGTC TGCACCAACG GCAGATAATG

                   360        370        380        390        400
>Anarabkdr  ---------- ---------- ---------- ---------- ----------
>Angambiae  AGACCAACAA GATTGCAGAA GCGTTCAACA GAATATCACG CTTTTCTAAC

                   410        420        430        440        450
>Anarabkdr  ---------- ---------- ---------- ---------- ----------
>Angambiae  TGGATTAAAA TGAATTTAGC AAACGCTCTC AAGTTTGTAA AAAATAAATT

                  460        470        480
>Anarabkdr  ---------- ---------- ---------- -------
>Angambiae  AACAAGCCAA ATAGCATCCG TTCAACCGAC AGGTAAA
```

Figure 2.10: Alignment of the *kdr* (susceptible) region from *An. arabiensis* and *An. gambiae*. The triplets encoding leucine (TTA) in both the susceptible *An. arabiensis* and *An. gambiae* mosquitoes are indicated in grey, arrows show positions of introns. Dashes are used to denote gaps introduced for maximum alignment.

2.4 Discussion

The results in this section indicate that a low level of physiological resistance to DDT in *An. arabiensis* has been developed under selective pressure in the laboratory. The LT50 and LT90 values of DDT increased significantly over 15

generations of selective pressure in both MAT and KGB *An. arabiensis* strains. In the selection of *An. arabienisis* MAT strain, high variation characterised the mortality values during generations F1 – F4 (Table 2.3) immediately after selective pressure was applied. This might have been due to the error made initially in the selection process by putting the survivors from the first two selection experiments back in the same cage with the parental colony. Mating between the two populations might have resulted in dilution of the selected resistant genotypes, therefore making the population more susceptible to DDT (Prasittisuk and Curtis, 1982). Alternatively, it has been suggested that high variation in mortalities is perhaps typical of populations in early stages of selective pressure (Theeraphap *et al*, 2002). The general patterns of the selection for DDT resistance are similar in both strains as depicted in (Figures 2.1 and 2.2). This result is typical of most laboratory regimes which tend to select within existing phenotypic distributions often at 80 – 90 % mortality in order to provide survivors for the next generation (Roush and McKenzie, 1987). The selective dose was closely controlled between 30 to 45 min and 30 – 60 min for the MAT and KGB respectively to permit discrimination among similar genotypes within the physiological distribution of phenotypes (Roush and McKenzie, 1987). However, in similar studies, 3 laboratory colonies G1, SENN and MBN of *An. arabiensis* have been selected for resistance to DDT at high doses and adults could survive exposure to DDT for 8 hours (Hemingway, 1981; Matambo *et al*, 2007). This suggests that the KGB and the MAT strains were at comparatively low level of DDT resistance. Theoretically, a susceptible colony composed of totally susceptible individuals will produce the highest slope for a regression line of dose – response data. With selective pressure from the exposures to insecticides, a population will become heterozygous for resistant genotypes and as the frequency of resistant genotypes increases, the slope of the regression line will shift to the right (Brown and Pal, 1971). There was a shift to the right in regression lines from dose response data for the populations under DDT selection in both the MAT and KGB strains (Figures 2.3 A and B) and the slopes of regression lines based on the data from these experiments continuously declined over time in the two strains (Table 2.4).

This result suggests that the resistance to DDT in the selected populations is not due to vigour tolerance but reflects true physiological resistance (Brown and Pal, 1971).

Evidence for cross resistance to permethrin was observed in the DDT – selected colony of *An. arabiensis* MAT strain (Figure 2.5). Previous studies have shown some evidence for cross – resistance in a G1 colony of *An. arabiensis* which has been selected for DDT resistance (Hemingway, 1981). In addition, pyrethroid resistance in *An. stephensi* larvae was reported in a strain that had developed DDT resistance as a result of selection experiments in Pakistan (Omar *et al*, 1980).The similar mode of action of DDT and pyrethroids can result in cross – resistance if mutations occur in the target site. No *kdr* mutations were found in either MAT or KGB colonies from this study (Figure 2.10). Similarly, low or no resistance to pyrethroid insecticides and DDT caused by the *kdr* mutation has been observed within the M form of *An. gambiae s.s* and *An. arabiensis* in several West African countries despite high levels of resistance being found within the S form of *An. gambiae* (Diabete *et al,* 2002). However, the absence of the *kdr* in the colony studied here is not conclusive considering the low number of specimens that were used in the assay and the inherent technical problems associated with the diagnostic PCR.

The results from this study have shown that more individuals with high GST activity are present in the selected than in the parental lines of both the strains, albeit at low frequency. This may be attributable to DDT selection pressure to which the mosquitoes in selected populations were continuously exposed. The order of magnitude of change in GST activity observed in the selected populations of MAT and KGB strains is similar to that seen in *An. arabiensis* field strains with GST – based DDT resistance (Hargreaves *et al*, 2003). This indicates the possible involvement of GSTs in conferring DDT resistance in MAT and KGB strains. Resistance to DDT has been associated with increased levels of GST activities in several species of mosquitoes including *An. subpictus*, (Hemingway *et al*, 1991) and *An. gambiae* (Parapanthadara et al., 1993). Elevated esterase activity was also detected in the MAT populations under DDT selection compared to the unselected population. The results in absolute unit for the alpha and beta subtrates were similar to those of Hargreaves *et al*, (2003). Casimiro *et al,* (2006), found lower average esterase activities with the two substrates in DDT susceptible populations of *An. arabiensis* from Mozambique. The monooxygenase activity is low in the selected populations in KBG strain, suggesting that the p450 enzyme system may not be not

be involved in DDT resistance in this strain. However, the monooxygenase activity is significantly higher in selected line compared to the parental line in MAT strain, but the small sample size is low and no conclusion could be made.

CHAPTER 3

ISOLATION OF EPSILON CLASS GST GENES FROM A LABORATORY STRAIN OF *AN ARABIENSIS*

3.1 Introduction

Glutathione S-transferases (GSTs) are soluble dimeric proteins that are found ubiquitously in living organisms. They are involved in the metabolism, detoxification and excretion of numerous endogenous and exogenous toxic compounds from the cell. More than 40 GSTs have been detected in the genomes of most higher eukaryotes for which the full genome sequence data are available (Holt *et al,* 2002). The majority of these GSTs have been classified into at least 13 different classes based on their amino acid sequence identities, immunological properties and, in some cases, substrate specificities (Board *et al,* 1997, 2002). Some of these, such as the Zeta and Omega classes, occur in a wide range of species, whereas the insect-specific Delta and Epsilon classes are more restricted in their distribution (Ranson *et al,* 2001). A comparative genome wide analysis between *D. melanogaster* and *An. gambiae,* revealed considerable expansion of the insect-specific classes of GSTs in the mosquito and this was consistent with differences in the ecology of these dipterans (Ranson *et al,* 2002). Hitherto, all the insect GSTs that have been implicated in xenobiotic metabolism belong to either the Delta or Epsilon classes (Ranson, *et al*, 2001). The Epsilon class GST gene cluster in *An. gambiae* has been mapped to chromosome 3R division 33C, at a position coinciding with a DDT resistance loci (Ranson *et al,* 2000b). Despite the taxonomic closeness of *An. gambiae* to *An. arabiensis*, the two species differ strikingly in their preferences for hosts, oviposition sites, spatial/temporal distributions and tolerance to man-made ecological changes (Coluzzi *et al,* 1979; Levine *et al*, 2004). The differences which GSTs may cause in the biology of these sibling species, has not been investigated.

Elevated levels of GST activity has been observed in a DDT – resistant field population of *An. arabiensis* from South Africa (Hargreaves *et al,* 2003) but the underlying mechanisms for this increased activity is not known. The preliminary results of the bioassay tests conducted on the laboratory colonies of *An. arabiensis,* have shown a low level of DDT resistance in the parental lines of MAT and KGB

strains. This prompted further selection of the colonies for higher levels of resistance to DDT. To investigate the molecular mechanisms conferring the DDT resistance, two approaches were employed; detection of the knock-down resistance (*kdr*) mutations and isolation of the Epsilon class *GSTe* genes. The diagnostic polymerase chain reaction (PCR) to detect the substitutions in the voltage sodium channel that had been previously detected in *An. gambiae* s.l. (Martinez-Torres *et al,* 1998; Ranson *et al,* 2000a) was used, but no such mutations were detected in the *An. arabiensis* colonies and the method was not pursued further.

The absence of the *kdr* alleles strongly suggests the presence of metabolic mechanisms causing resistance to DDT and the biochemical data suggests potential involvement of *An. arabiensis* GST genes. To date there are no published sequences for *An. arabiensis* GSTs and no sequences were found in the NCBI database, searching using the keywords GST and *An. arabiensis.* Therefore, primers which were designed for amplification of the individual members of Epsilon class *An. gambiae* GSTs were used to amplify the orthologous genes from *An. arabiensis.* The isolated *An. arabiensis* GST genes were compared to those of *An. gambiae* in terms of phylogenetic relationships, amino acid similarity and intron/exon sizes.

3.2 Materials and Methods

3.2.1 Mosquito Strains

Mosquitoes from the colonies of the MAT strains of *An. arabiensis* were reared in the laboratory as described in sections 2.2.1.1 and 2.2.1.2.

3.2.2 Mass Genomic DNA Extraction

Genomic DNA was extracted from approximately 1g of pooled batches of *An. arabiensis* MAT strain as described previously (Vaughan and Hemingway, 1995). The mosquitoes were homogenised under liquid nitrogen and added to 15 ml extraction buffer, which contained EDTA and 20µg/ml pancreatic RNase A. The mixture was mixed gently in an SS34 tube (Sarstedt). The suspension was incubated at 37°C for one hour for RNA digestion. Proteinase K (Sigma) was added to a final concentration of 100µg/ml. The homogenate was incubated at 50°C for five hours and

then 0.35 volumes of saturated sodium chloride were added. The mixture was chilled on ice for five minutes, followed by centrifugation at 4°C for 20 minutes at 16,000g in an RC5C refrigerated centrifuge (Sorvall Instrument Dupont). The supernatant was transferred to a fresh 50 ml tube and an equal volume of isopropanol was added and mixed to precipitate the DNA. The pellet of gDNA was then washed with 2ml of ice-cold 70% (v/v) ethanol and centrifuged at 4°C for 20 minutes at 16,000g. Additional cleaning of the DNA was performed using phenol-chloroform protocol and precipitation by ethanol. The DNA was air-dried and re-suspended in 1ml TE buffer pH 8.0.

3.2.3 Extraction of Total RNA

Two batches of four individual one-day old adult mosquitoes each from the MAT and KGB strains were homogenized in 400µl TRI reagent (Sigma). The samples were centrifuged at 12,000g for ten minutes at 4°C to remove the debris. The supernatant was transferred to a DEPC-treated centrifuge tube and was left at room temperature for five minutes to ensure complete dissociation of the nucleo-protein complex. Eighty micro litres of chloroform was added and the mixture shaken vigorously for 15 seconds and incubated at 4°C for 10 minutes. The sample was centrifuged at 12,000g for 15 minutes at 4°C after which the colourless upper aqueous phase, containing RNA, was transferred to a fresh tube. To precipitate the RNA, 0.2ml of molecular grade isopropanol was added. The sample was mixed and incubated at room temperature for 10 minutes. After centrifugation at 12,000g for 10 minutes at 4°C, the pellet was air-dried and re-suspended in 26µl of nuclease-free water. Total RNA was treated with RQ1 DNase (Promega) to degrade any remaining DNA in the presence of RNasin (Promega).

3.2.4 Synthesis of 1st Strand cDNA

cDNA was synthesized from total RNA using superscript T^{M111} RNase H^- reverse transciptase (Invitrogen) and an oligo (dT) adapter primer (5′-GACTCGAGTCGACATCGA $(dT)_{17}$ – 3′). One microgram of RNA was mixed with 1µg of the primer and heated to 65°C for five minutes to dissociate the secondary conformation. The reaction mixture was first chilled on ice for 1 minute and then pre-warmed to 50°C for 2 minutes after addition of 8µl of 5X first strand buffer (250mM

Tris-HC1 pH 8.3, 375mM KCL, 15mM $MgCl_2$), 2µl of 10mM of each dNTP and 2µl of 0.1M DTT. One half microlitre of Superscript T^{M111} Rnase H^- reverse transcriptase was added and the reaction was incubated at 50°C for 90 minutes. The reaction was then heated to 70°C for 15 minutes to inactivate the enzyme. First strand cDNA samples were stored at -20°C.

3.2.5 Amplification of Epsilon GST genes in *An. arabiensis*

Primer pair sequences designed for the amplification of individual members of the *An. gambiae* Epsilon class GST genes (Ding, *et al.,* 2003) (Table 3.1) were used to amplify the full-length orthologous genes from *An. arabiensis.* PCR products were amplified using gDNA and cDNA as templates. The PCR reactions contained 1.5mM $MgCl_2$, 0.2mM of each dNTPs, and 0.5 µm of each primer, 1 X reaction buffer (Qiagen) and 1.25 units of Taq DNA polymerase (Hotstar) in a final volume of 25µl. Annealing temperatures ranging from 50°C – 60°C were determined empirically for each gene using a gradient PCR machine PTC-200 (MJ Research). The cycling parameters were 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C – 60°C for 30 seconds and 72°C for 30 seconds and a final extension of 72°C for 10 minutes. The PCR products were separated on a 2% (w/v) agarose gel in the presence of ethidium bromide (0.5µg/ml) and then visualized using the Gene Genius Bio Imaging system (Syngene).

Table 3.1: Primer sequences used for amplification of *An. arabiensis* Epsilon Class GSTs

Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Expected band size (bp)
GSTe1	ATC CCG AAA CCT GTG CTA TA	CGC TCT TGC AGC GAG TCA CTT	817
GSTe2	ATC ACC GAG AGC CAC GCA ATC A	GCA CCG TTC GCT CCC TCG CAG T	830
GSTe3	ATG GCA CCG ATT GTG TTG TAC	CTA AAC CTT GCC AAG TTT CTG G	741
GSTe4	CGC CAT TCA AAC GAC CAT GCC	CTC AAC CAG ATC AGG TTC AC	743
GSTe5	ATG GCA ACG AAC CCC ATC ATC	CCT TGG AAT CGT GGC TTT AC	765
GSTe6	GTA AGC TCT ACA GCT CGT TCG GC	GCG AAG ATC AAC CGG TAG TGG TG	774
GSTe7	CGA GAT TGG TAC TGT ACA CG	CTC GGA TAG AGA CCG TCG TC	819
GSTe8	TTA GTA CCG GCC TGT ACT ACG AC	CTA CAG CTT AAT TCT TTC CCG CT	789

3.2.6 Subcloning of PCR products into P^{GEM}-T Easy Vector

3.2.6.1 Ligation of PCR products into P^{GEM}-T Easy Vector

The PCR products of expected sizes were ligated into a P^{GEM}-T Easy Vector ligation system (Promega). To get a 3:1 insert:vector molar ratio, the following equation was used to estimate the amount of PCR product that was required in the ligation reaction:

$$\frac{25\text{ng vector} \times \text{size of insert in bp}}{\text{size of vector in bp}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

Each reaction contained 25ng of P^{GEM}-T Easy Vector and 3 units of T4 DNA ligase. The reactions were incubated at 4°C for 24 hours.

3.2.6.2 Transformation of E. coli JM109 subcloning grade competent cells with plasmid DNA

The JM109 competent cells (Invitrogen) were thawed on ice. Fifty microlitres of cells were added into a pre-chilled, 15ml Falcon™ tube 2059 polypropylene tube (Becton Dickinson). Two microlitres of the ligation reaction were added to the cells and the tube was incubated on ice for 20 minutes. The plasmid was introduced into the competent cells by heat-shock in a 42°C water bath for 45 seconds. After incubating the tube on ice for 2 minutes, 0.9ml of pre-heated SOC medium at 42°C (2% (w/v) bacto-tryptone, 0.5% (w/v) yeast, 0.05% (w/v) NaCl and 20mM glucose), was added into each tube, which was then incubated at 37°C for 30 minutes with shaking at 250 rpm. One hundred microlitres of transformed bacteria were then plated on agar plates containing 100µl 2% (w/v) X-gal (5-bromo-4chloro-3indolyl-beta-D-galacto pyronoside [Bioline]) and 10µl of 0.1M 1PTG (isopropyl – beta-D-thiogalacto pyronoside (Sigma). The plates were incubated overnight at 37°C.

3.2.6.3 Identification of positive colonies by PCR screening

The P^{GEM}-T Easy Vector contains a lac Z gene which encodes an enzyme capable of metabolizing B-galactosidase to give a blue colour. If an insert is successfully cloned into the vector it interrupts the lac Z gene and stops the production of the enzyme resulting in white colonies. Positive (white) colonies were selected using sterile yellow tips, inoculated onto fresh LB-agar plates containing 50µg/ml ampicillin and a

small portion deposited in a sterile PCR tube. The LB-agar plate was incubated overnight at 37°C. PCR reactions each contained 1.5mM $MgCl_2$, 0.2mM of each dNTP, 0.5mM of M13 forward (-5′ GTT TTC CCA GTC ACG AC-3′) and reverse (-5′ CAGGAAACAGCTATGAC-3′) primers and 2.5 units of DNA polymerase in the reaction buffers from Bioline. These M13 primers bind to sites in the vector flanking the multiple cloning site and are used to ascertain if a band of correct size has been inserted. The PCR conditions were 95°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and finally 72°C for 10 minutes. PCR products were separated on a 2% (w/v) agarose gel and sized by comparing with DNA ladder standard.

3.2.6.4 Culture of selected transformation colonies

Individual colonies containing a PCR insert of the expected size were inoculated using a yellow sterile tip into 2ml of LB-broth (selected yeast 5g, tryptone 10g and sodium chloride 5g per litre) containing 50µg/ml Ampicillin. These cultures were incubated overnight at 37°C, 250 rpm in an orbital incubator.

3.2.6.5 Plasmid purification

Bacterial culture (1.5ml) was centrifuged at 14,000 g for one minute at room temperature. Plasmid DNA was isolated using the QIA Prep Miniprep Kit (Qiagen). The pelleted bacterial cells were re-suspended in 250µl buffer P1 (containing 0.1mg/ml RNase Alkaline lysis was conducted by adding 250µl of buffer P2 and mixing gently by inverting the tube. Buffer N3 (350µl) was added to neutralize the alkaline and the tube was inverted immediately but gently 4-6 times. The solution was centrifuged for 10 minutes at 14,000g to separate cell debris from the plasmid supernatant. The supernatant was transferred to the QIA Prep column and the plasmid DNA was bound to the column due to the high salt binding condition from buffer N3. The column was centrifuged for one minute 14,000g and the flow-through discarded. After washing the column with 0.75ml buffer PE (Binding buffer) to get rid of contaminants, plasmid DNA was eluted with 30µl Elution buffer (100mM Tris-HCl,

pH 8.5) and allowed to stand for one minute. The plasmid DNA was collected from the column after centrifugation at 14,000g for one minute.

3.2.7 DNA Sequencing

Plasmid DNA was sequenced in the forward and reverse directions using the M13 primers. The sequencing was performed using the dye terminating sequencing method and the products were electrophosed on a Beckman Coulter CEQ8000. Analysis of the sequences was done using the DNA Star package software (Lasergene). Identity of the sequences obtained with related sequences in the database of the National Institute of Health, USA (http://www.ncbi.nih.gov/Blast) by searching using the BLAST program.

3.2.8 Phylogenetic Analysis

Putative amino acid sequences of the GSTs were aligned using Clustal W (Thompson et al 1994). The alignment was converted to PHYLIP file using TREECON software (Van de Peer and De Watcher, 1993). Phylogenetic trees were determined by neighbour-joining methods (Saitou and Nei, 1987) using the TREECON software (Van de Peer and De Watcher, 1994).

3.3 Results

3.3.1 Identification of *An. arabiensis* GST genes

Degenerate PCR was performed using primers designed for *An. gambiae* GST genes to amplify orthologous genes in *An. arabiensis*. The full length genomic and cDNA sequences of each of the eight GST orthologs of the Epsilon class GST gene cluster on chromosome 3R of *An. gambiae* were amplified from *An. arabiensis* MAT strain. The sizes of PCR products obtained for both genomic and complementary DNA of each of the eight *An. arabiensis* GSTs are shown in Table 3.2. Cloning of the PCR products and sequencing at least 3-5 clones for each *An. arabiensis* GSTs gene yielded sequences with high percentage identity when compared to *An. gambiae* GSTs. The percentage similarity at the amino acid level between the eight *An. arabiensis* GST proteins and their equivalents in *An. gambiae*, ranged from 91% to 98.7% (Table 3.2).

The putative amino acid sequences of *An. arabiensis* GSTs were aligned using Clustal W. An alignment of the deduced amino acid sequences of the eight genes identified in this study is shown in Figure 3.1. The invariant residues indicated in bold letters are conserved in all the eight proteins and the underlined motifs, which characterised Epsilon class GSTs, are conserved in these and all previously identified orthologous genes in *An. gambiae* (Ortelli *et al,* 2003).

Table 3.2: Comparison of Epsilon class *An. arabiensis* GSTs with orthologous genes in *An. gambiae*

Gene	Product size (bp)		Percentage identity of amino acid sequence (with Ag) with *An. gambiae*	Number of introns (intron size bp) *An. arabiensis*	Number of introns (intron size bp) *An. gambiae*
	cDNA	Genomic			
GSTe1	675	817	94.7	2 (64 and 78)	2 (64 and 78)
GSTe2	624	788	98.6	2 (74 and 90)	2 (74 and 90)
GSTe3	745	814	98.7	1 (69)	1 (69)
GSTe4	693	758	98.7	1 (65)	1 (65)
GSTe5	709	787	97.0	1 (78)	1 (72)
GSTe6	692	782	97.8	1 (90)	1 (91)
GSTe7	681	821	91.2	2 (80 and 60)	2 (69 and 66)
GSTe8	658	774	97.7	2 (50 and 66)	2 (66 and 60)

The table shows the PCR products sizes of both the genomic and cDNAs from each member of *An. arabiensis* as well as the number and sizes of introns identified in individual genes. The number of introns in the orthologous genes identified in *An. gambiae* is shown (Ortelli, 2002).

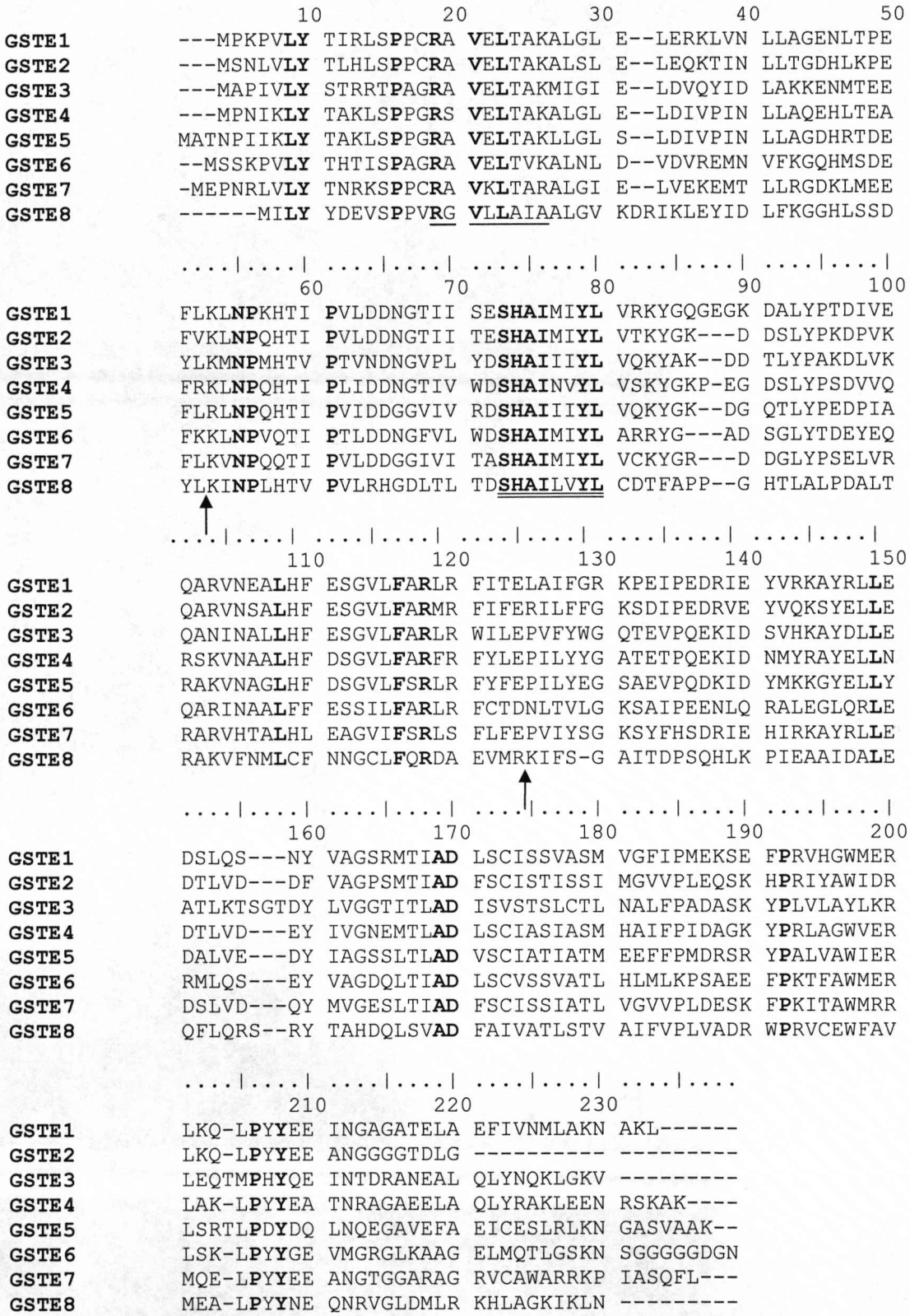


Figure 3.1 Clustal W alignment of deduced amino acid sequences of *An. arabiensis* GST gene cluster, arrows indicate positions of introns. The number and positions of introns are conserved compared to *An. gambiae*.

Sequences shown are from the MAT strain. Residues in bold are conserved in all eight proteins. The two underlined residues are motifs characteristic of Epsilon GSTS (single underlined) or most GST classes (double underlined).The arrows indicate intron positions.

3.3.2 Intron positions and sizes

The cDNA sequences of *An. arabiensis* GSTs were aligned with the genomic DNA sequences and the positions and sizes of introns were noted. The coding sequence of all the eight genes is interrupted by an intron or two introns in *An. arabiensis*. The first intron is formed at 48 amino acid residues from the N- terminal in all the eight Epsilon *An. arabiensis* GST genes (Figure 3.2). Intron sizes ranged from 50bp in *GSTe8* to 90bp in *GSTe6* and *GSTe2*. The number of introns in each gene is the same as that found in its orthologous counterpart in *An. gambiae*, but there are some slight variations in the sizes (Table 3.2). The intron –exon boundaries of the genomic sequences, illustrated by *GSTe2*, are consistent with the canonical GT/AG rule (Figure 3.2). The positioning of these introns within the coding sequences are highly conserved when compared between *An. gambiae* and *An. arabiensis.*

```
M  S  N  L  V  L  Y  T  L  H  L  S  P  P  C  R  A  V  E  L  T  A  K  A  L  S  L  E  L  E  Q  K  T  I
ATGTCCAACCTTGTACTGTACACCCTGCACCTTAGCCCACCGTGCCGTGCCGTGGAGCTGRCGGCCAAAGCRTTGRGCTTGGAGCTGGAGCAGAAGACCA

  N  L  L  T  G  D  H  L  K  P  E  F  V  K                    Intron 1
TTAATCTGCTGACGGGTGACCATTTGAAGCCGGAATTTGTGAAggtacgtaatgggattgagagagaamgttagaaagamagcgattggtatgcattaca

                  L  N  P  Q  H  T  I  P  V  L  D  D  N  G  T  I  I  T  E  S  H  A  I  M  I  Y  L  V
ttacccwtaygtgcacagCTAAACCCGCAACATACGATCCCGGTGCTGGATGACAACGGTACGATCATCACCGAGAGCCACGCGATCATGATCTATCTGG

  T  K  Y  G  K  D  D  S  L  Y  P  K  D  P  V  K  Q  A  R  V  N  S  A  L  H  F  E  S  G  V  L  F  A
TGACGAAGTATGGCAAAGATGATAGCCTCTATCCAAAAGACCCCGTCAAGCAGGCCCGTGTAAATTCGGCCCTGCACTTCGAGTCCGGCGTACTGTTCGC

 R  M  R  F  I  F  E                                    Intron 2
CCGGATGAGATTCATTTTYGtaagtgacgtgacctggttttcccttaaaaagactgagaccggttccagttccagcataacgccaagcattttccaaacc

                  R  I  L  F  F  G  K  S  D  I  P  E  D  R  V  E  Y  V  Q  K  S  Y  E  L  L  E  D  T  L
ccttccacaggAACGTATCCTATTCTTCGGCAAATCGGACATCCCCGAGGATCGCGTTGAGTACGTGCAGAAATCGTACGAGCTGCTGGAGGACACACTG

V  D  D  F  V  A  G  P  S  M  T  I  A  D  F  S  C  I  S  T  I  S  S  I  M  G  V  V  P  L  E  Q  S  K
GTGGACGACTTTGTCGCCGGACCGAGCATGACGATTGCCGACTTTAGCTGCATTTCCACGATCTCGAGCATTATGGGTGTGGTGCCGTTGGAGCAGTCGA

  H  P  R  I  Y  A  W  I  D  R  L  K  Q  L  P  Y  Y  E  E  A  N  G  G  G  G  T  D  L  G  K  F  V  L
AGCATCCCCGGATCTACGCGTGGATCGATCGGCTGAAGCAGCTGCCCTACTACGAGGAAGCGAACGGTGGCGGAGGCACCGATCTGGGCAAGTTTGTGCT

 A  K  K  E  E  N  A  K  A  *
AGCCAAAAAGGAGGAGAATGCTAAGGCTTAA
```

Figure 3.2 Genomic and deduced amino acid sequences of An. arabiensis *GSTe2* isolated by PCR from MAT strain. The positions of introns are indicated in lower case letters. Intron 1 found 48 amino acid residues from the N – terminal is conserved compared to *An. gambiae*

3.3.3 Polymorphisms in *An. arabiensis* GSTs

The sequences obtained from the various clones which were derived from each of the *An. arabiensis GSTe1 – GSTe8*, were arranged into their respective contigs using the Seqman programme DNA star package. The DNASP software was used to identify polymorphic sites in the nucleotide and derived amino acid sequences of each gene. Nucleotide ambiguities resulting from sequencing of multiple clones were observed with genomic and cDNA sequences. The number of polymorphic sites in amino acid and nucleotide sequences in and between An. arabiensis and *An. gambiae* GSTs are shown (Table 3.3). Of the eight GST genes, *GSTe1* has the highest number of nucleotide substitutions (22), of which 12 are non-silent mutations, resulting in changes in the amino acid residues. Two polymorphic sites were identified in each of the putative amino acid sequences of *An. arabiensis GSTe1* and *GSTe2* (Table 3.3). Figure 3.3 shows the amino acid differences between *An. arabiensis GSTe1* protein and the allelic variants *GSTe1K* and *GSTe1Z* in *An. gambiae*. The *An. arabiensis* protein *GSTe1* is closer to the Kisumu variant *GSTe1K*, differing in seven amino acids than the Zanu *GSTe1Z* with which it differs by 12 amino acids

Table 3.3: Comparison of polymorphic sites within and between *An. arabiensis* and *An. gambiae* GSTs.

	A. arab vs A. g (Zan)		A.arab vs A.g.(Kis)		Kis vs Zan		*An arabiensis*	
	A.acid	**Nucl**	**A. acid**	**Nucl**	**A. acid**	**Nucl**	**A.acid**	**Nucl**
Genes								
GSTe1	12	19	7	12	12	23	2	2
GSTe2	2	8	na	na	na	na	2	5
GSTe3	2	3	0	0	2	2		
GSTe4	5	7	1	2	6	8	1	3
GSTe5	5	12	5	18	0	13	1	3
GSTe6	3	6	15	10	0	0	-	-
GSTe7	17	18	15	10	4	10	-	-
GSTe8	4	10	na	na	na	--		-

Analysis of polymorphic sites in the deduced amino acid (A.acid) and cDNA (Nucl) sequences of Epsilon class *GSTe1 – GSTe8* gene clusters from *An. arabiensis* and *An. gambiae.* Consensus sequences from 3 – 5 clones for each of the *An. arabiensis* GSTs and sequences for orthologous genes from *An. gambiae* Zanu (Zan) and Kisumu (Kis) strains (retrieved from the database) (Genebank) were used for the analysis (na = no sequence available).

```
gste1           MPKPVLYTIRLSPPCRAVELTAKALGLELERKLVNLLAGENLTPEFLKLNPKHTIPVLDD
gste1Kis.       --------V----------------------------Q--------------------
gste1Zn         --------VH---------------------------------------------------

gste1           NGTIISESHAIMIYLVRKYGQGEGKDALYPTDIVEQARVNEALHFESGVLFARLRFITEL
gste1Kis.       -------------------------------E--------------------------
gste1Zn         -------------------------------D--------------------------

gste1           AIFGRKPEIPEDRIEYVRKAYRLLEDSLQSNYVAGSRMTIADLSCISSVASMVGFIPMEK
gste1Kis.       ----------------------------TD------L--------------------R
gste1Zn         VF-A--------------T-----------D----------------------------R

gste1           SEFPRVHGWMERLKQLPYYEEINGAGATELAEFIVNMLAKNAKL
gste1Kis.       ---------------------------------------------
gste1Zn         ---------I-M----------------------D--------
```

Figure 3.3: Shows the amino acid differences between gste1 proteins in *An.arabiensis* MAT and *An. gambiae* Zn. and Kis. strains.
The dashes indicate where the amino acids are identical in all 3 strains.

3.3.4 Phylogenetic analysis

To examine the relationship between *An. gambiae* and *An. arabiensis* Epsilon class GST genes, multiple sequence alignment of gene clusters from each species was carried out prior to molecular phylogenetic analysis. The *An. gambiae* Epsilon class GST gene sequences were retrieved from Genebank. Deduced amino acid sequences of the 16 GSTs, eight each from *An. arabiensis* and *An. gambiae,* were aligned using clustal W and a phylogenetic tree was constructed using TREECON (Figure 3.4). The dendogram illustrates the close phylogenetic relationship between the gene families from the two species. Each gene in the cluster is tightly associated with its ortholog. To further investigate the relationship between *An. arabiensis* GSTs and those from other insects, all the known insect-specific Epsilon GST sequences from *Drosophila melanogaster* and *Ae. aegypti*, in addition to those of *An. gambiae*, were retrieved from Genebank and aligned using clustal W. The alignment, consisting of all the *An. arabiensis* GST subunits and 25 putative Epsilon class GSTs from the three species, were used to construct phylogenetic tree by distance neighbour-joining method. A representative distance tree generated from the alignment is shown (Figure 3.5). The consensus phylogenetic tree further indicated the *An. arabiensis* Epsilon class GST genes to be more closely related to equivalent families in *An. gambiae* and *An. aegypti*, but distantly related to those in *D. melanogaster.*

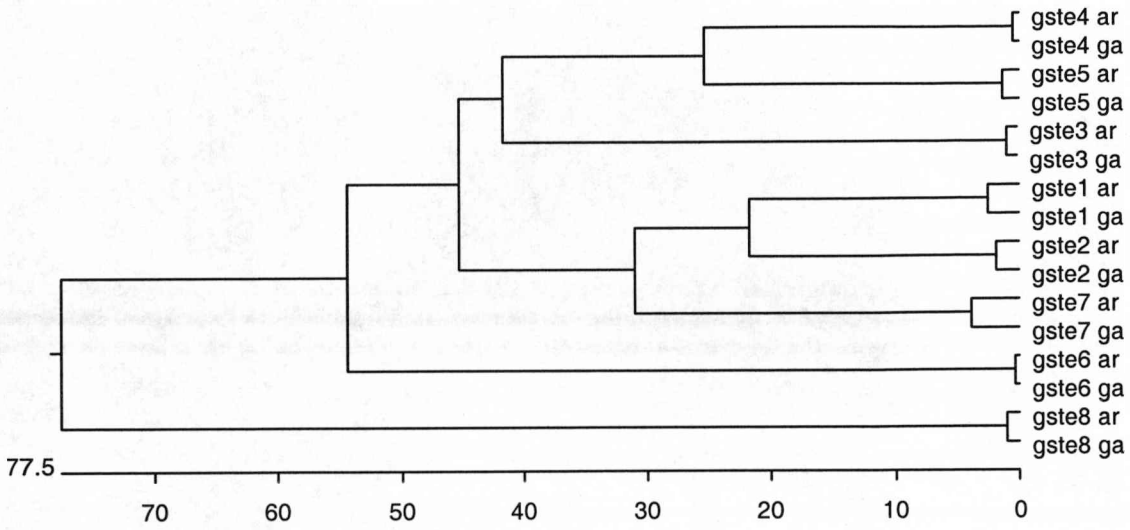


Figure 3.4: Dendogram illustrating the relationship between *An. arabiensis* and *An.gambiae* Epsilon class GSTs.

Amino acid sequences were aligned using Clustal W and the tree was constructed with the neighbour – joining method program from a similarity matrix of pairwise comparisons made by using the jukes – cantor algorithm. The program assumes a constant evolutionary rate, the bar indicates the frequency at which 0.1 amino acid substitution occur at any given site.

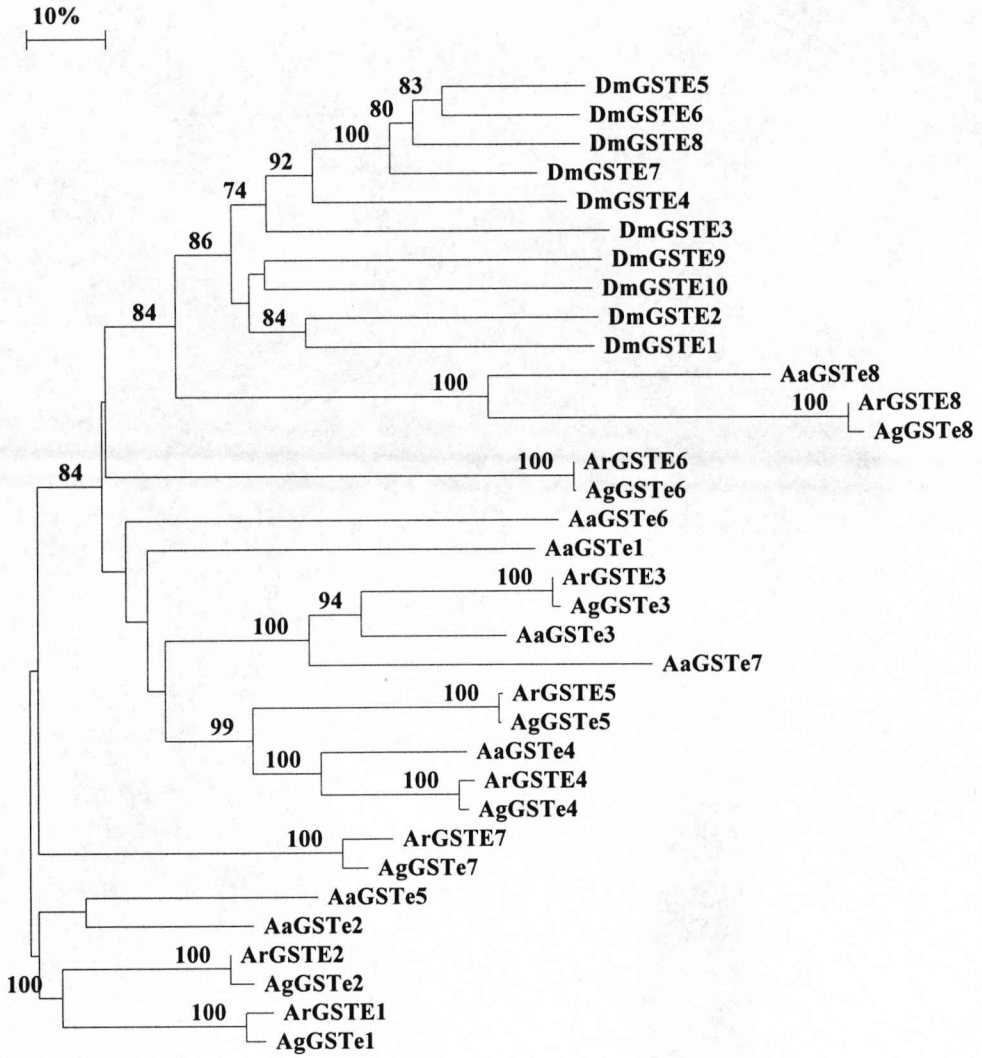


Figure 3.5: Phylogenetic relationships of Epsilon GST classes in insects (Dm = *Drosophila melanogaster*, Aa = *Aedes aegypti*, Ag = *An. gambiae,* Ar = *An. arabiensis*)

Distance neighbour – joining tree illustrating the relationship between *An. arabiensis* , *An. gambiae*, *Aedes aegypti* and *D. melanogaster* Epsilon class GSTs. Clustal W was used to align the putative amino acid sequences of 8 Epsilon class GST subunits in *An. arabiensis* with 25 putative epsilon class GSTs from *D. melanogaster*, *Ae. Aegypt*i and *An. gambiae*. The tree was constructed by the neighbour – joining method from a similarity matrix of pairwise comparisons made using the jukes – Cantor algorithm. Nodes with distance bootstrap values (500 replicates) of > 70% are shown.

3.4 Discussion

3.4.1 Identification of *An. arabiensis* GSTs

Given the important role of GSTs in the detoxification of environmental xenobiotics (David *et al*, 2000 a b), it was postulated that different members of the *An. gambiae* complex, which differed in their ecological adaptations may differ in their pattern of GST expression. The focus was on the Epsilon GSTs because of the strong evidence establishing the role of some members of the class in conferring DDT-based metabolic resistance in *An. gambiae* (Ranson *et al,* 1997, 2001). To date, all research investigating this extensive gene family in the *Anopheles gambiae* complex is centred on *An. gambiae* s.s. To study the *An. arabiensis* GSTs, primers which were designed for *An. gambiae* GSTs were used to isolate, by PCR, the orthologous genes from the laboratory colony of *An. arabiensis* MAT strain.

Eight GSTs were identified in this work from *An. arabiensis* MAT. No GSTs had been previously identified from this species and therefore pest sequence could not be retrieved from the EST database for comparison. However, these *An. arabiensis* GST genes aligned closely with the Epsilon class GST genes, which have been already identified in *An. gambiae* (Ranson *et al,* 2002, Ortelli *et al,* 2003). The motifs which are characteristics of Epsilon class GSTs, including the semi-conserved motifs shared by other GST classes, are 100% conserved in all the eight *An. arabiensis* GST genes. The high percentage similarity (91.2% - 98.7%) at amino acid level, which was observed between *An. arabiensis* GSTs and the identified Epsilon GSTs in *An. gambiae,* is well above the 40% cut off point criteria which was used for classifying a gene to a particular class (Chelvanayagam *et al,* 2001). This strongly suggests the inclusion of the *An. arabiensis* GST into the Epsilon class. The introns found in *An. arabiensis* GSTs are similar in respect of size, number and positions to those reported in their corresponding orthologous genes in *An. gambiae* (Ding *et al,* 2003). The considerable conservation of introns in the Epsilon GST gene clusters between the two sibling species is indicative of their common ancestry and supports the evidence shown in a molecular phylogenetic study of the *Anopheles gambiae* complex (Besansky *et al,* 1994).

3.4.2 Polymorphisms in *An. arabiensis* GSTs

At chromosomal level, inversion polymorphisms have been identified as the genetic basis for the biological traits characterising the *An. gambiae* complex as the most efficient malaria vector system in the world (Coluzzi, 1992). *An. arabiensis* is polymorphic for inversions a and c on chromosome 2R, which were shown to be associated with non-uniform, indoor resting and feeding behaviour, and this was interpreted to mean decreased chances of exposure to indoor insecticide-treated surfaces (Coluzzi *et al,* 1979). Nigatu et al., (1995) reported an association of DDT resistance with the 2Rb inversion, which was explained to be presumably due to presence of a resistance gene in the inverted chromosome. In this study, polymorphisms in the GST sequences were observed within the *An. arabiensis* MAT and between it and the Kisumu and Zanu strains of *An. gambiae*. The majority of the substitutions within *An. arabiensis* strains are silent mutations and, therefore, this is assumed to reflect allelic variations that are maintained in the colony. However, a more detailed analysis of the *An. arabiensis* GST gene sequences is required to determine whether the mutations are attributable to allelic variation or may be due to recent gene duplication, as was suggested in *An. gambiae Cyp4* P450 genes (Ranson *et al,* 2002). Nevertheless, out of the eight *An. arabiensis* GSTs, *GSTe1* is observed to be the most polymorphic. The deduced amino acid and nucleotide sequences of this gene were compared to those of *GSTe1*K and *GSTe1Z*, the two highly polymorphic variants of the orthologous gene in *An. gambiae* (Ortelli *et al,* 2003). Surprisingly, the proteins from the *An. gambiae* differed between themselves by 12 amino acids, while the *An. arabiensis GSTe1* protein differed by only 7 amino acids with the Kisumu *GSTe1*K variant. This result supports the finding that the Kisumu variant, unlike the rarer alleles, exists at high frequencies in natural population and may confer some fitness advantage under field conditions (Ortelli *et al,* 2003).

3.4.3 Phylogenetic analysis

A preliminary phylogenetic analysis was conducted to establish the relationship between the GSTs isolated from *An. arabiensis* and all the known Epsilon class GSTs in *An. gambiae*. At this stage the full extent of the GST gene family and their organisation in the genome of *An. arabiensis* was not known. The initial analysis (Figure 3.4) shows the two gene families forming a tight cluster with orthologous genes from both species pairing with one another. The dendogram supports evidence

of a close relationship between these two species, as was reported (Besansky *et al,* 1994). A further assessment of the phylogenetic relationships of *An. arabiensis* GSTs with other insect GSTs supports this view. The topology of the tree in Figure 3.5 clearly separates the Culicidae GST gene lineage from the Drosophilidae GSTs as indicated by the respective bootstrap values. Similarly, within the mosquito GST gene families, the anopheline GSTs, except *GSTe3* and *GSTe4* are paired and separated from their evolutionary more distant *Aedes* counterparts (Krzywinski *et al,* 2001). There are no clear orthologs between these mosquito GSTs and the Drosophilia lineage supporting the earlier suggestion that the Epsilon class GSTs from these dipteran families have radiated independently (Ranson *et al*, 2002). The close orthology of *An. arabiensis* GST gene with those of *An. gambiae* might indicate similar *in vivo* biological functions of the individual genes as patterns of orthology was believed to provide clues to physiological functions (Ranson *et al,* 2002).

The results in this chapter describe the cloning and identification of eight *An. arabiensis* GSTs. There are no previous studies on GSTs from this species, but the alignments and phylogenetic analysis suggest placement of the *An. arabiensis GSTs* into the insect Epsilon class of GSTs. However, the full extent of the GST gene family in *An. arabiensis* can be explored only when the genome sequence becomes available, but the biochemical and immunological properties of the recombinant proteins of some of the genes, particularly *An. arabiensis GSTe1* and *GSTe2* identified in this work, can be studied further to confirm the classification

CHAPTER 4

QUANTIFICATION OF GST EXPRESSION DURING DEVELOPMENT IN *AN. ARABIENSIS*

4.1 Introduction

During their life-cycle, *Anopheles* mosquitoes occupy diverse habitats and feed on various diets encountering harmful substances that must be rapidly detoxified. The adults feed on plant nectar and mammalian blood which is rich in potentially toxic substances such as heme, while the larval diet often contains bacterial toxins and plant allelochemicals (Wallace and Merritt, 2004). Anopheline mosquitoes may encounter insecticide treated surfaces when searching for human host or resting indoors after feeding (Curtis, 1996) and their breeding sites may be contaminated with pesticides that are used to control crop pests (Service, 1977; Ijumba, *et al*, 2001). The broad spectrum of xenobiotic compounds which the mosquitoes encounter have necessitated the development of extensive families of detoxification enzymes such as GSTs (Ranson and Hemingway, 2005). In mosquitoes, the expression patterns of those individual GSTs which have been studied varied markedly between developmental stages and were found to be influenced by previous exposure to insecticides. Investigating the life – stage expression profile of *GSTe2* in three strains of *Aedes aegypti*, Lumjuan *et al,* (2005), reported the highest expression level in larvae in the DDT resistant PMD-R strain. Five out of eight Epsilon class GSTs were shown to be constitutively expressed at high levels in one – day old adults in *An. gambiae* (Ding *et al,* 2003). Recently, a whole organism transcriptome approach, using microarray was used to determine the expression profile of the detoxification genes in adults, larvae and pupae of *An. gambiae* (Strode *et al,* 2006). The highly specific microarray chips contained unique fragments from *An. gambiae* detox genes including 35 GSTs. They reported a complex pattern of changes in gene expression, with approximately 25% of the genes being constitutively expressed at significantly different levels in the three life – stages.

For example, the results showed that four GST genes including *GSTe1*, *GSTe2*, *GSTd1-4* and *GSTd1-1* were expressed at 2 – fold higher level, in larvae than adults, while the Sigma GST variants, *GSTs1-1* and *GSTs1-2* were expressed at significantly higher levels in adults than in larvae (Strode *et al*, 2006).

In the sibling species *An. arabiensis*, which shares similar ecological habitats to *An. gambiae,* very little is known regarding the developmental expression of these detoxification genes.

In the previous chapter, the isolation of eight *An. arabiensis* Epsilon class GST genes was described. In this chapter, the basal expression of three of the GSTs, *GSTe1*, *GSTe2* and *GSTe4*, was investigated in the larval, pupal and adult stages of two strains of *An. arabiensis*, which differed in their susceptibility to DDT. To further assess the effect of exposure to insecticides on the expression of each of the three *An. arabiensis* GST genes, the experiments were repeated with mosquitoes from the KGB selected line. This line of mosquitoes has been maintained on selection pressure with 4% DDT for over 20 generations. Quantitative real-time PCR was used to quantify the transcripts of the three *An. arabiensis* Epsilon class GST genes in the life-stages of the susceptible and resistant strains. The initial copy number of each of the GST transcripts in the cDNA sample was quantified by comparison with standard curves, which were generated using serially diluted plasmids containing full-length cDNA from each of the Epsilon GST gene. The ribosomal protein gene, SP7 was used to normalise for variation in concentration of the templates.

4.2 Materials and Methods

4.2.1 Mosquito Strains

The mosquitoes used in this study were taken from the parental lines of the MAT and from the parental and selected KGB strains. Adult mosquitoes from the parental MAT strain are more susceptible to DDT compared to those from the parental and selected lines of the KGB strain, as described in Chapter 2. A set of three biological replicate samples was taken from the larval, pupae and adult stages of the mosquitoes from the two strains. Each set contained a batch of ten individual specimens randomly sampled from each life-stage.

4.2.2 Sequencing of GST Genes

The full-length sequences of *GSTe1*, *GSTe2* and *GSTe4* were amplified as described in Chapter 3, using cDNA from the susceptible MAT, and DDT-resistant KGB, strains. The PCR products were ligated into the P^{GEM} –T-Easy Vector and transformed into *E. coli* JM109 competent cells (Promega).

4.2.3 Quantitative PCR on cDNA

4.2.3.1 cDNA synthesis

Total RNA was extracted from 3 replicate samples (10 mosquitoes per replicate) of 4^{th} instar larvae, pupae and one-day old adults (5 males and 5 females) from each of the three lines of both strains. The procedure for extraction and cDNA synthesis is described in Chapter 3.

4.2.3.2 Plasmid construction

The *GSTe1*, *GSTe2* and *GSTe4* standard plasmids were constructed by insertion of full-length cDNA from the MAT and KGB strains into the P^{GEM}-T Easy Vector as described in Section 3.2.2. The ribosomal protein gene SP7, used as an internal control plasmid, was amplified using primers SPC and SPD (Table 4.1) and ligated into the P^{GEM}-T Easy Vector. The concentrations of the plasmids were determined using an ND-1000 spectrophotometer (Nanodrop Technologies).

4.2.3.3 Quantitative PCR and standard curve preparation

The *GSTe1*, *GSTe2*, *GSTe4* and *SP7* standard plasmids were diluted serially to concentrations ranging from 1 fg/µl to 1ng/µl. Quantitative PCR was used to quantify and compare the abundance of the transcripts from each of the 3 GST genes in the different life-stages of the MAT and KGB strains. In all cases the primers were designed to span an intron as a control against genomic DNA contamination, and for each of the GST genes, the primers were selected to avoid regions of allelic variation within the gene sequences (Table 4.1). Strain-specific primers were used for amplifying *GSTe1* and *GSTe4* genes from the MAT and KGB because of the nucleotide differences at the primer binding sites between the variant gene sequences from the strains. The plasmid and cDNA templates were amplified using the

Quantitative SYBR Green PCR Kit (Qiagen). SYBR Green 1 is a double-strand DNA specific dye, which binds to the minor groove of the DNA helix during amplification cycles. Quantitative PCR was performed by amplifying 1µl of cDNA with 0.5µM of each primer and 1 x SYBR Green Master Mix in a final volume of 15µl. The conditions used for the amplification of each of the three Epsilon GST and the *SP7* genes are specified in Table 4.1. The PCR reaction was conducted for 35 cycles. Plasmid DNA standards and negative controls were included in the same plate, for each experiment. Three biological replicate samples from each strain and stage in the life-cycle were used as templates. A sample was analysed in duplicate in each experiment and results were averaged from three separate experiments. The incorporation of the SYBR Green 1 into the PCR products was quantified using the DNA Engine Opticon (MJ Research). The fluorescence was plotted in relation to the number of cycles and the crossing line was produced to obtain the standard concentration related to the cycle number. A straight line standard curve was obtained by plotting the cycle number against the logarithmic value of the standard concentration.

4.2.3.4 Gene copy number calculation

The initial copy number for each of the Epsilon GSTs was calculated automatically by measuring the fluorescence produced due to incorporation of the SYBR Green 1 dye into the double stranded PCR product. The value was then compared to the standard curve produced from the PCR amplification of the fragment of the particular gene from plasmids of known concentrations. From the sequences of the P^{GEM}- T Easy Vector and cDNA fragments that were inserted into the vector, the molecular weights (MW) of the plasmids used to produce the standards were calculated. The mRNA copy number of each transcript was calculated using the following equations:-

Copy number/fentogram = Moles/fg of plasmid x Avogadro's constant.

For instance, the number of copies of *GSTe2* per fentogram was calculated as shown below:-

Moles/fg of plasmid = Molecular weight of *GSTe2* +Molecular weight of P^{GEM} –T Easy Vector

Full-length of *GSTe2* = 624 bp

A + T = 278 x 697 = 193766 g/mole

C + G = 346 x 712 = 246352 g/mole

= 440118 g/mole

Molecular weight of T-Easy Vector = 2131255 g/mole

Total molecular weight of plasmid = 2571373 g/mole

Therefore Moles/fg of plasmid $= 10^{-15}/2571373$

$= 3.889 \times 10^{-22}$

Copy number/fg $= 3.889 \times 10^{-22} \times 6.023 \times 10^{23}$

= 234.2 copies/fg

The length of each Epsilon GST and SP7 cDNAs, the AT and GC contents, the molecular weights of the plasmids and the initial copy number per fentogram of each Epsilon GST plasmid are given in the Table 4.2.

Table 4.1: Primers used in quantitative real-time PCR

Gene	Primer Name	Primer Sequences (5′ to 3′)	cDNA size	Annealing	Extension	Reading
GSTe1	*GSTel*qAF *GSTe*lqAR *GSTel*AKR	GTC AAT GAG GCA CTG CAC TTC GTG ATC CGG CTA CGT AAT TG AGC TCA GGT CAG CGA TCG TC	180	60°C for 30 seconds	72°C for 30 seconds	82°C
GSTe2	*GSTe2*Qaf 150HGSTR	ATC ACC GAG AGC CAC GCG ATC A GCC ACG GTT CGC TTC CTC GTA GTA	280	60°C for 30 seconds		
GSTe4	15BGST 160HR *GSTe4*qAKR	CGC CAT TCA AAC GAC CAT GCC GAT GGC GTG GCT GTC CCA CAC G CAT TGA TGG CGT GGC TGT CC	229	60°C for 30 seconds		
SP7	SPC SPD	GCA CGT CGT GTT CAT TGC CG GAA CAT TAA CGT CAC GGC CAG TCA	291	56°C for 30 seconds		85°C

*GSTe1*AKR and *GSTe4*qAKR are the reverse primers used for amplifying *GSTe1* and *GSTe4* from the KGB strain

Table 4.2: Calculation of the initial copy number of Epsilon GST cDNA plasmids

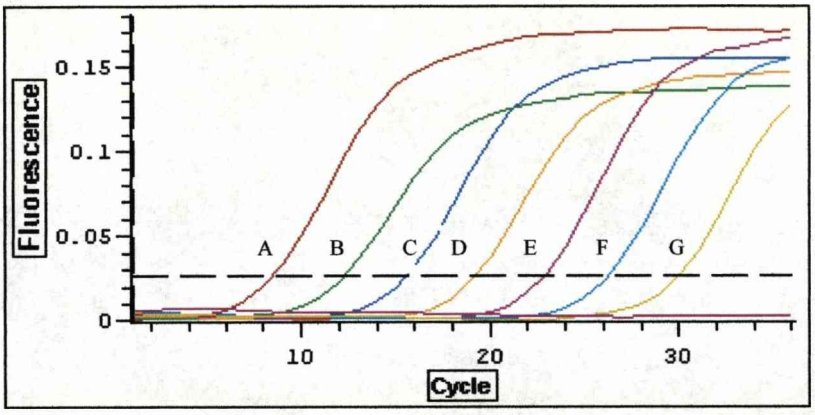
Genbank name	Length of cDNA (bp)	AT content	GC content	Molecular weight of full-length cDNA	Molecular weight of Plasmid	Initial copy No/fg
GSTe2	624	278	346	440118	22571373	234.2
GSTe1	675	313	362	475905	2607160	231
*GSTe1*K	676	294	382	475478	2606733	231
GSTe4	693	294	399	488309	2619564	236.4
*GSTe4*K	697	291	406	491899	2623154	236
SP7	489	232	257	344688	2475943	241.3

***GSTe1*K and *GSTe4*K are plasmids containing the genes amplified from KGB strain**

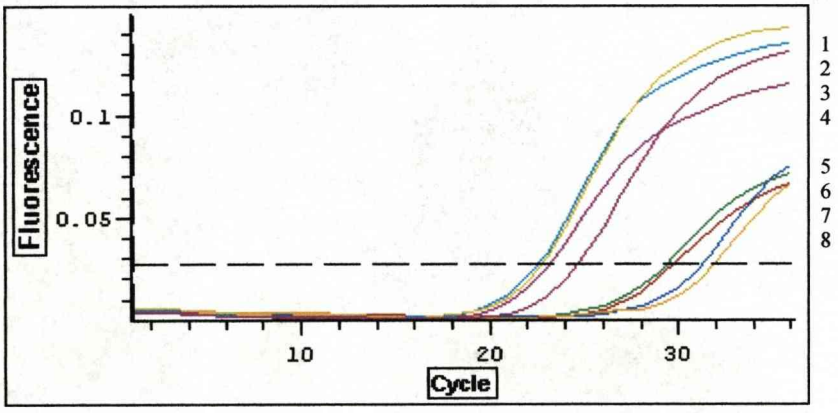
4.3 Results

The differential expression profile of GSTs across developmental stages has been documented in *An. gambiae*. To assess the developmental expression pattern of Epsilon GSTs in the sibling species *An. arabiensis* and the effect of DDT resistance on the expression, the messenger RNA copy number of the *An. arabiensis* Epsilon GSTs genes in different life-stages of MAT, KGB-P and KBG-R strains was determined. These three lines of *An. arabiensis* differed significantly in their response to DDT. The resistance ratio between MAT and KGB-P is 1.5 and the KGB-R has been selected for DDT resistance up to 20 generation (see Chapter 2). The *An. arabiensis* Epsilon *GSTe1*, *GSTe2* and *GSTe4* were analysed since their orthologues are significantly over expressed in DDT resistant ZAN/U compared to susceptible Kisumu strain in *An. gambiae* (Ding *et al*, 2003). Complementary DNA from 3 biological replicate samples each taken from a batch of ten individuals were used as templates in quantitative real – time PCR. Three developmental stages: larvae (L), pupae (P), and adult (A) were assessed. A sample was analysed in duplicate in three independent experiments. From the standard curves shown in Figure 4.1 (A), it was possible to extrapolate the fluorescence values obtained with the cDNA samples in each life stage as illustrated in Figure 4.1 (B) The results obtained from the three separate experiments were averaged for each life – stage. The mRNA copy numbers were determined by comparison with known concentrations of standard plasmids and normalized against the copy number of the ribosomal *SP7* gene. Expression of all three genes (*GSTe1*, *GSTe2* and *GSTe4*) was detected in all life stages (Table 4.3, Figures 4.2 – 4.34).

The expression levels of the genes followed very positively skewed (log-Normal) distributions, so were transformed to natural logarithms for statistical analysis. Results are thus presented as geometric (detransformed log) means with their 95% confidence intervals (Table 4.3).



A



B

Figure 4.1 Quantification of *GSTe2* mRNA expression levels in Larvae and Adults *An. arabiensis* KGB strain.

(A) SYBR Green 1 fluorescence acquisition by PCR products from serially diluted (1 ng to 1fg) standard plasmids and (B) biological replicates cDNA samples each extracted from 10 randomly selected individual larvae and adults from KGB selected line. 1 – 4 represent replicates larval samples and 5 – 8 adult samples

The X – axis represent the cycle number and the Y – axis indicates the value of the fluorescence acquisition. A to G in (A) indicate the concentrations ranging from 1 ng/µl to 1 fg/ul in the serially diluted plasmids containing the *GSTe2* insert.

Table 4.3: Quantitative PCR results of *An. arabiensis GSTs*

Gene	Life stage	Normalized cDNA copy numbers					
		MAT	(95% CI)	KGB - P	(95% CI)	KGB -R	(95% CI)
GSTe1	Larvae	0.137±0.02	(0.123 – 0.152)	0.614±0.06	(0.575 – 0.655)	0.618±0.12	0.536 -.0.701
	Pupae	0.023±0.023	0.019 – 0.28	0.132±0.02	(0.120- 0.146).	0.03±0.01	(0.024 – 0.058)
	Adult	0.036±0.0.1	(0.031 – 0.041)	0.586±0.11	(0.521 – 0.658)	0.566±0.10	(0.485 – 0.660)
GSTe2	Larvae	0190±0.06	(0.152 – 0.237)	1.249±0.23	(1.125 – 1.387)	2.426±0.55	(1.988 – 2.962)
	Pupae	0.115±0.01	(0.110 – 0.119)	0.507±0.10	(0.451 – 0.569)	0.635±0.0.09	(0.575 – 0.701)
	Adult	0.172±0.04	(0.148 – 0.199)	0.584±0.11	(0.516 – 0.661)	0.938±0.42	(0.672 – 0.764)
GSTe4	Larvae	0.066±0.03	(0.045 – 0.096)	0.151±0.02	(0.134 – 0.171)	0.091±0.02	(0.075 – 0.109)
	Pupae	0.038±0.01	(0.03 – 0.046)	0.306±0.07	(0.265 – 0.355)	0.026±0.07	(0.021 – 0.052)
	Adult	0.049±0.01	(0.042 – 0.058)	0.036±0.01	(0.030 – 0.042)	0.135±0.07	(0.098 – 0.018)

The GST transcript copy number was determined by normalizing with transcript copy number of ribosomal *Sp7* transcript.

Differences were evaluated for each gene separately. One-way analyses of variances were applied initially to test for differences across all three life stages in all three lines simultaneously; significant results were then investigated in detail using the Turkey"honestly significant difference" multiple comparison test (a less conservative variant of the Bonferroni procedure) to protect against spurious significance due to multiple testing (Winter, 1962). Statistical significance was set at the conventional 5% level for all tests. The expression of individual genes was compared between developmental stages within and between strains as follows:

GSTe1 expression levels

The observed levels of *GSTe1* expressed at each life stage, the geometric means and 95% confidence intervals for these observations are shown for each species separately in Figure 4.2.

In the MAT strain the mean levels differed significantly in larvae compared to adult and pupae ($p < 0.001$ for all comparisons). The lowest average level was found at the pupae stage. The mean level at the adult stage was approximately 50% higher, while the mean level at the larvae stage was 5.5 times greater than at the pupae stage. The expression of *GSTe1* in the KGB-P strain showed mean levels at the larvae and adult stages to be both significantly greater than that at the pupae stage ($p < 0.001$), but the larvae and adult stages did not differ significantly ($p > 0.999$). The mean levels at the larvae and adult stages were approximately 4.5 times greater than at the pupae stage. In the KGB-R line, again, mean levels of *GSTe1* at the larvae and adult stages were both significantly greater than that at the pupae stage ($p < 0.001$), but the larvae and adult stages did not differ significantly ($p = 0.997$). The mean levels at the larvae and adult stages were approximately 20 times greater than at the pupal stage.

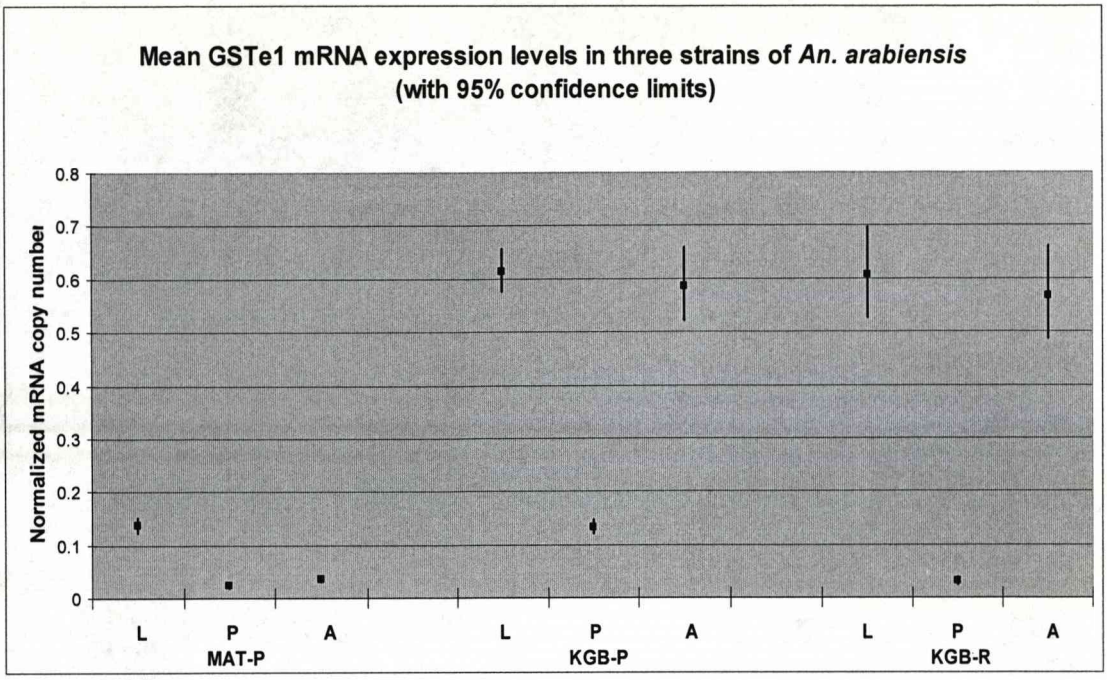


Figure 4.2: Geometric mean *GSTe1* mRNA expression levels in three strains of *An. arabiensis* (with 95% confidence limits).

GSTe2 expression levels

For *GSTe2* the observed levels of expression, the geometric means and their 95% confidence intervals for these observations are shown in Figure 4.3. In the MAT –P strain the mean levels (≤ 0.19), at the larvae and adult stages were both significantly greater than that at the pupae stage ($p < 0.001$), but the larvae and adult stages did not differ significantly ($p = 0.977$). The mean levels at the larvae and adult stages were around 60% greater than at the pupae stage. For the KGB strain, in the KGB – P line, the mean level of *GSTe2* at the larvae stage was 2 and 2.5 times greater than the means at the pupae and adult stages respectively – both differences were highly significant ($p < 0.001$). However, the difference between the pupae and adult stages was not statistically significant ($p = 0.849$). In KGB-R line the mean level at the larvae stage was 3.8 and 2.6 times greater than at the pupae and adult stages respectively – both differences were highly significant ($p < 0.001$). The mean level at the adult stage was approximately 50% higher than that at the pupae stage – this difference was significant, but at a lower level ($p = 0.010$).

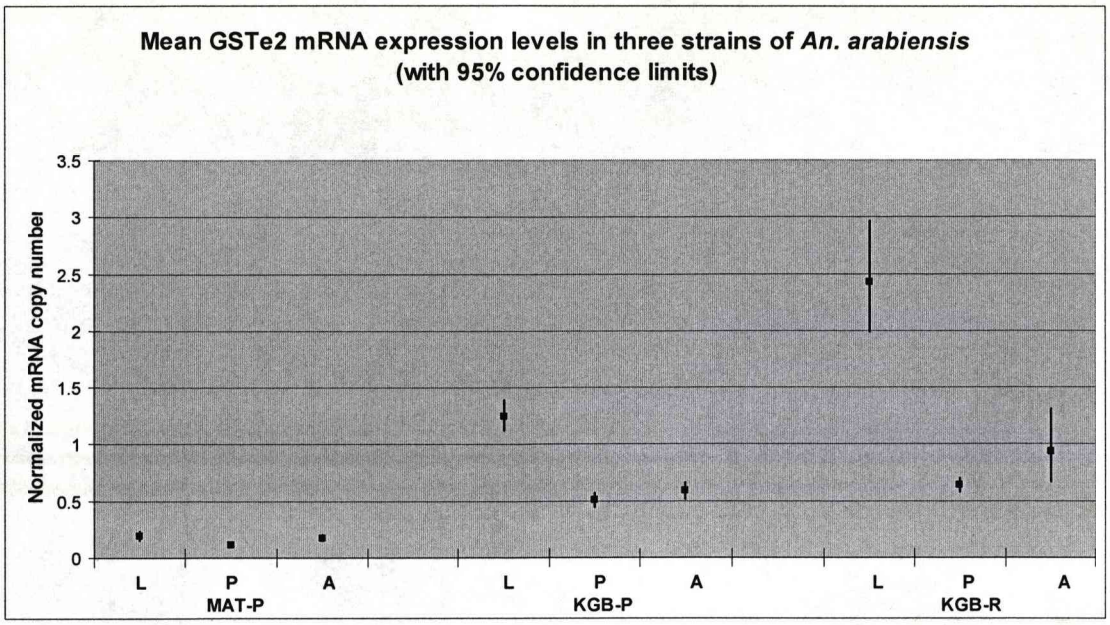


Figure 4.3: Geometric mean *GSTe2* mRNA expression levels in three strains of *An. arabiensis* (with 95% confidence limits)

GSTe4 expression levels

The geometric means and 95% confidence intervals for the observed levels of *GSTe4* expressions at each life stage are shown for each strain in Figure 4.4. The mean level at the larval stage was 75% greater than that at the pupal stage – this difference was statistically significant ($p = 0.021$) in the MAT -P. However, no significant difference was found between the larvae and adult stages ($p = 0.525$), or between the pupal and adult stages ($p = 0.763$) in this species. In KGB strain, the mean levels at the larval and pupal stages were 4.2 and 8.6 times greater than the mean at the adult stage respectively – both differences were highly significant ($p < 0.001$) in the KBP - P. And the mean level at the pupae stage was approximately double the mean level at the larvae stage ($p = 0.001$). In KGB-R line, the mean levels at the larvae and adult stages were 3.5 and 5.1 times greater than at the pupal stage respectively – both differences were highly significant ($p < 0.001$). The mean level at the adult stage was approximately 50% higher than that at the larval stage – but this difference was not statistically significant ($p = 0.176$).

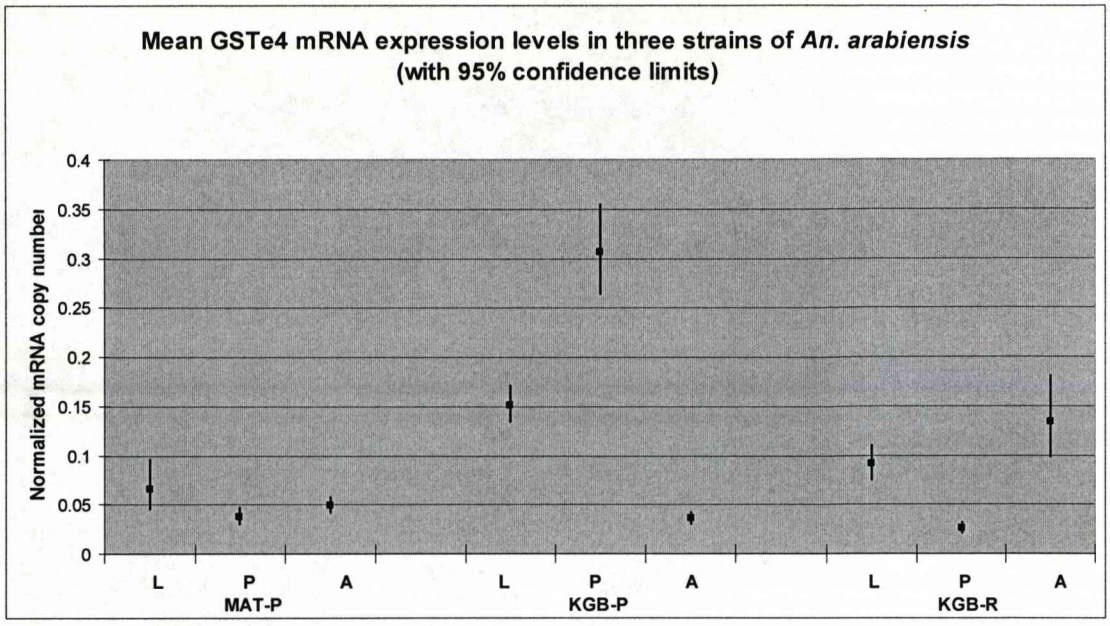


Figure 4.4: Geometric mean *GSTe4* mRNA expression levels in three strains of *An. arabiensis* (with 95% confidence limits)

Comparative expressions of GSTs in *An. arabiensis* strains

After testing for significant differences in levels of the expressions of the individual genes across life stages within strains, significant results were analysed further using the Turkey "honestly significant difference". This is a multiple comparison test which protects against false significance due to multiple testing. The relative expressions of each of the three genes in all the life stage in the strains is shown (Table 4.4)

Table 4.4: Comparative expressions of GSTs in developmental stages between strains.

Gene	Life stages	RATIO OF KGB-P/MAT	COPY KGB-R/MAT	NUMBER KGB-R/KGB-P
GSTe1	Larvae	4.5***	4.5***	1
	Pupae	5.7***	1.3	0.2
	Adult	16.2***	15.7***	0.96
GSTe2	Larvae	6.5***	12.8***	1.9***
	Pupae	4.4***	5.5***	1.2
	Adult	3.4***	5.5***	1.6**
GSTe4	Larvae	2.2***	1.4*	0.6
	Pupae	8.8***	0.7	0.8
	Adult	0.7	2.8***	3.8***

The transcript copy number of each GST was determined by normalizing with transcript number of ribosomal *SP7* gene. The ratio of the average copy is obtained by comparing the resistant with the susceptible in each pair. Statistical differences were evaluated using the Turkey "honestly significant difference" multiple comparison test (a less conservative variant of the Bonferroni procedure). Statistical significance was set at the conventional 5% level for all tests ($p < 0.001$ indicates by *).**

4.4 Discussion

The expression patterns of *An. arabiensis* Epsilon *GSTe1*, *GSTe2* and *GSTe4* genes were investigated in developmental stages of the MAT – P and the two lines (KGB- P and KGB-R) of the KGB strain. The expression levels of the individual genes were measured at mRNA levels. *GSTe1* was expressed at higher levels in larval and adult stages than in pupae in all the strains. The consistent high expression of this gene in the larvae and adults may indicate its involvement in protecting the *Anopheles* mosquito against stressful effect of dietary and environmental chemicals to which it may be exposed at these stages of the life – cycle (Diapoulous *et al,* 2002). The ortholog *AgGSTe1* in *An. gambiae* posseses peroxidase activity and can protect cells against oxidative stress (Ortelli *et al*, 2003).

GSTe1 was over expressed 4.5 fold in larvae and 16 fold in adult in the KGB – P and KGB – R compared with the MAT – P, suggesting a positive correlation with resistance status of the strains. Recombinant *GSTE1* does not metabolize DDT, but it is possible the enzyme may be conferring some resistance to the secondary effects of insecticide exposure. GSTs with peroxidase activity have been implicated in pyrethroid resistance in a resistant strain of plant hopper (Vontas *et al*, 2002). However, whether *GSTe1* in *An. arabiensis* possess peroxidase activity remains to be investigated. The positive correlation of the expression of *GSTe1* is not reflected in the KGB – P and KGB – R lines, in both of which, the mean levels of the expression in larvae and adults did not differ significantly ($P > 0.999$). Strode *et al*, (2006) have reported higher expression level of *GSTe1* in larvae than in pupae and adult in *An. gambiae*.

GSTe2 was significantly over expressed in larvae and adult ($P < 0.001$) than in pupae in all strains. The orthologs of *GSTe2* in the mosquitoes *Aedes aegypti* and *An. gambiae* were also at higher levels in larvae than in later developmental stages (Lumjuan *et al*, 2005; Strode *et al*, 2006). Relative to the MAT –P strain, mean expression levels of *GSTe2* were 6.6 and 12.8 times greater in KGB – P and KGB – R ($p < 0.001$ for both comparisons). A positive correlation of high *GSTe2* transcript with resistance status is indicated by the higher expression in KGB – R compared to KGB – P line ($p < 0.001$).

However, in this study, only the adult stage was selected with DDT and it is not known if the resistance observed in this life stage was conferred at the larval stage also. Nevertheless, the mechanism responsible for up regulation of *GSTe2* in larvae of *An. arabiensis* KGB – R line is unknown. Out of the three *An. arabiensis* GST genes that were investigated in this study, *GSTe2* was over expressed at consistently higher levels relative to *GSTe1* and *GSTe4* in all the life stages as has been reported (Strode et al. 2006). The high expression of this gene suggests that it is ubiquitously expressed in mosquito tissues and may indicate a general house keeping or biosynthetic role in addition to its involvement in specialized detoxification pathways.

In contrast, the expression of *GSTe4* fluctuated in all the strains in different life stages and without definite correlation with insecticide resistance status. For example, in the KGB –P, the gene was expressed at higher levels in the larvae and pupae than in adult

but the expression was 3.5 and 5.1 times greater in larvae and adult stages than in pupae in the KGB R line. The *Aedes aegypti* ortholog, *AeGSTe4* was also expressed at significantly high level in pupae and adult in parental PMD but not in PMD – R strain (Lumjuan et al. 2005). However, in this study, the mean expression level of *GSTe4* in adult stage was significantly higher in the KGB – R strain than in MAT – P and KGB – P ($p < 0.001$), but the MAT – P and KGB – P strains did not differ significantly ($P = 0.394$). The mechanisms responsible for the regulation of these genes at developmental stages of *An. arabiensis* need to be investigated.

The knowledge on the susceptibility or detoxification capacities of different developmental stages is required to determine the stage at which resistance monitoring test should be directed.

CHAPTER 5

INDUCIBLE EXPRESSION AND TRANSCRIPTIONAL REGULATION OF EPSILON *GSTE2*

5.1 Introduction

The term induction refers to the process in which a chemical stimulus enhances the activity of a detoxification system by the production of additional enzymes (Terriere, 1984). Induction of detoxification systems was first investigated intensively in higher animals because of its important implications for drug and xenobiotic metabolism (Nerbert *et al*, 1981). In the insect, three important systems of detoxification, including monooxygenases, carboxylesterases and glutathione S-transferases have been implicated in metabolism of various insecticides (Scott, 1999; Hemingway and Ranson, 2000; Hemingway *et al*, 2004).

Expression of GSTs is induced by oxidative stress in rats and the increased expression of these enzymes results in a decrease in the levels of ROS (Hayes and Pulford, 1995). The general patterns of GST expression in many insect species are affected by various dietary compounds, insecticides and laboratory inducers and have been comprehensively reviewed (Clark, 1989; Yu, 1996). Different levels of induced GST activity have been reported by treatment of the Fall Armyworm *Spodoptera frugiperda,* and the Southern Armyworm, *Spodoptera eridiana*, either with plant diet or allelo chemicals (Yu, 1982b; Bratten *et al,* 1984). Increases of GST activity of up to 18-fold, as a result of exposure of *Spodoptera frugiperda* to indole 3-acetonitrile, an active chemical derivative of plant diet, was reported (Yu, 1984). It has also been shown that the dietary induced GST activity caused an increase in tolerance to the insecticides diazinon and methyl parathion (Yu, 1982a). Non-lethal doses of pesticides and related chemicals are shown to have substantial effects on the expression of GST in the insect species. Exposure of the honey bee, *Apis mellifera,* to sub-lethal doses of permethrin caused 2-fold increase in the GST activity with respect to DCNB (Yu, 1984). However, the insecticides of other classes such as carbaryl, malathion, methoxychlor and dibenzflouron, were ineffective in this regard. Induction of GST activity in the housefly, *Musca domestica*, has been demonstrated after treatment with non-lethal levels of a variety of insecticides (Hayaoka and Dauterman,

1982). Of those tested, DDT was the most potent, causing a 50% increase in GST activity compared to dieldrin, methyl paraoxon and parathion. Capdevilla *et al,* (1973), reported that DDT dehydrochlorinase in a DDT-resistant housefly strain was increased by about 50% after exposure to non-lethal doses of DDT. In most of the cases a model substrate, such as 1-chloro-2,4 dinitrobenzene (CDNB) was used to assay GST activity from crude insect homogenate and the results, therefore, were measurements of the activity of a large subset of GSTs and do not reveal much about fluctuations in the levels of individual enzymes. Hence, previous studies on induction in insects have dwelt largely on the toxological and biochemical levels and only recently have the molecular aspects been considered. Le Goff *et al,* (2001) have demonstrated that the expression level of *GSTd1* in *Drosophila* increased approximately 3-fold, 72 hours after treatment with phenobarbital. Recently microarray approaches have been used to compare the differences in the global expression of genes constitutively and after insecticide exposure of insecticide resistant and susceptible strains of *An. gambiae* (Vontas *et al,* 2005). A short-term exposure of the pyrethroid-resistant RSP strain to permethrin induced expression of many genes, some belonging to enzyme families such as peptidases and UDP-glucoronosyl transferases that are not usually associated with insecticide resistance. Using quantitative PCR, Ding *et al,* (2005) found significant increases in the transcripts of the individual Epsilon GST genes after exposure to H_2O_2 (*GSTe1*, 4-fold, *GSTe2*, 1.7-fold and *GSTe3*, 3.6-fold).

It has been suggested that over-expression of GSTs could result from transcription factors, activated by external stimuli or chemical compounds bound to specific cis-regulatory element, which induce the transcription of GST genes. It was also proposed that changes in trans - acting elements that act to repress the transcriptional activity of GSTs regulate the over-expression of GSTs in *Aedes aegypti* (Grant and Hammock, 1992). The molecular mechanism underlying the inducible transcription of *An. gambiae* GSTs in response to oxidant treatment is not known. However, putative transcription factors such as NF-Kb, AhR and Forkhead transcription factor were identified in the *GSTe2* and *GSTe3* promoter regions in *An. gambiae*, but their role in the induction of these genes was not clarified (Ding *et al,*. 2005).

In the previous chapter (Chapter 4), three Epsilon GST genes were shown to be constitutively expressed in each of the life stages of the KGB and MAT strains of *An. arabiensis*. This chapter reports on the induction of *GSTe2* by permethrin, H_2O_2 and DDT. To assess the effects of these xenobiotic compounds on the expression of Epsilon *GSTe2*, larvae were exposed to sub-lethal doses of each of the xenobiotics and the expression of *GSTe2* was monitored by quantitative PCR. Putative regulatory elements controlling basal and induced the transcription were identified in the promoter regions of the *GSTe2*.

5.2 Materials and Methods

5.2.1 Induction of Epsilon *GSTe2* expressions by DDT, permethrin and H_2O_2

5.2.1.1 Mosquito and chemicals

Mosquitoes from the parental lines of MAT and KGB laboratory strains of *An. arabiensis* were used in the induction experiments. The parental lines of these strains were selected for the induction studies because both strains have been maintained in the insectary for a least 3 (MAT) and 2 (KGB) years, without any insecticidal selection pressure. When tested for susceptibility to DDT, adult KGB mosquitoes were more tolerant to DDT compared to MAT (Chapter 2). The susceptibility of the larvae of these mosquitoes to permethrin and DDT is not known. The choice of the inducing chemicals was based on the need to simulate the chemical contamination in the mosquito's natural breeding habitats. Hence, H_2O_2, permethrin and DDT were chosen as representative xenobiotic compounds some of which *An. arabiensis* may encounter in the environment. Previously, sub-lethal doses of 3mM H_2O_2 (Ding *et al,* 2005) and 0.02 mg/L of permethrin (David, personal communication) were used to treat larvae from ZAN/U and RSP strains of *An. gambiae*, therefore these dosages were adopted in this experiment. In order to determine the sub-lethal dose of DDT, 3 batches each of 25 larvae were exposed, each for one hour, to the following concentrations of DDT, 0.5 mg/L, 0.05 mg/L and 0.005 mg/L. Larval mortalities were determined after 24 hours holding. The dose 0.05 mg/L, which gave a mortality of >20%, was adopted and used for subsequent induction experiments.

5.2.1.2 Recruitment of larvae for induction

Rearing activities were harmonised to minimise any compounding effect, possibly due to excess food or overcrowding. Egg batches laid by the adult females were collected from filter paper placed in the egg-cup. The filter paper containing eggs was carefully cut to split the egg-batch into two halves. Each half of paper was placed in an egg pot containing 10-20 mls of water to enable the eggs to hatch to 1st instar larvae. About 25-30 1st instar larvae were transferred to shallow trays measuring 28 x 20cm with a depth of 6.5 cm, which were only half filled with distilled water. All larvae from the two strains were fed on Tetramin fish food flakes, using ground-up flakes for the first instars. Distilled water was applied to the larval trays on a daily basis to aerate the water and excess food was removed using a pipette. The water was changed if there were any signs of cloudiness, along with sluggish swimming behaviour of the larvae. Extreme precaution was taken to avoid contamination between the strains and lines of *An. arabiensis.* Pipettes and egg pots were colour-coded for each strain. This controlled method of larval rearing was used to generate all the 4th instar larvae which were used for all the induction experiments.

5.2.1.3 Exposure of 4th instar larvae to H_2O_2, permethrin and DDT

A total of six hundred 4th instar larvae from each of the MAT and KGB strains were exposed to H_2O_2, permethin and DDT in a series of separate experiments. In each experiment, a group of 40-50 larvae were immersed for one hour in either 3 mM H_2O_2, 0.02 mg/L permethrin or 0.05 mg/L DDT. The larvae were then transferred to distilled water to recover. Unexposed larvae of the same life-stage from both strains were used as control. Larval samples in batches of 10 were collected in triplicate at one, two and 24 hour time points post exposure (DDT) and at 2, 5, and 24 hour for permethrin.

5.2.1.5 Quantitative PCR of cDNA of *GSTe2*

To compare the abundance of *GSTe2* transcript in the treated and control samples in both strains, quantitative real time PCR was performed. Details of the primer sequences and the quantitative PCR conditions are presented in Table 4.1. The incorporation of the fluorescent dye SYBR Green during PCR amplification was detected using an OPTICON quantitative PCR machine (DNA Engine Opticon, M J

Research, USA). In each experiment, duplicate reactions were set up for the standard plasmids and the cDNA samples. Quantitative PCR was performed in 15 µl reactions with a quantitative PCR kit (Qiagen), each reaction comprised of 1µl of DNA template, 1 x Master Mix, 0.2 mM of each primer. The normalised copy number was determined for each sample as described earlier (Section 4.2.1).

5.2.2 Analysis of *An. arabiensis GSTe2* and *GSTe*1 intergenic regions

The term integenic region in this thesis is used to refer to the distances between the coding region of *An. gambiae* Epsilon GST genes, including untranslated region of neighbouring GST genes ranging from 175 bp to 1200 bp. The intergenic region upstream of *GSTe1* and *GSTe2* in *An. gambiae*, are 352 bp and 752 bp respectively (Figure 1.2).

This section investigates the potential regulatory roles of homologous regions of the genome in *An. arabiensis*. Sequences upstream of E2 in the KGB, MAT and field samples were compared to find mutations that may explain differences in the expression levels of GST genes observed in the two strains. The promoter elements were compared to those found in *An. gambiae*.

5.2.2.1 Extraction of nucleic acids

Genomic DNA was extracted as described above (Section 3.2.2) from three batches of male and female one-day old adult mosquitoes, each from the parental lines of MAT, KGB and field collected samples of *An. arabiensis*.

5.2.2.3 Amplification of E1 and E2 intergenic spaces

To amplify the E2 and E1 intergenic regions, species-specific primers (Table 5.1) were designed using the Primer 3 software. The intergenic regions were amplified from 100 ng genomic DNA in 25 µl PCR reaction volume with 0.5 mM of each primers, 1.5 uM $MgCl_2$, 0.2 mM of each dNTPS and 2.5 units of Taq DNA polymerase in the manufacturer's buffer. Cycling conditions were 95°C for 15 minutes, followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 45 seconds, 72 °C 10 minutes. The products were ligated into P^{GEM} T-Easy Vector (Promega) transformed into JM 109 competent cells (Stratagene) and screened by

PCR as described previously (Section 3.2.7 – 3.2.7.5) Plasmid DNA was extracted using the QIA prep spin miniprep (Qiagen) and submitted for sequencing on a Beckman CEQ 8000 (Beckman). A total of six different colonies from each strain containing each insert were sequenced in both forward and reverse orientations.

Table 5.1: Primers used to amplify E1 and E2 intergenic regions in *An. arabiensis*

Intergenic region	**Primer name**	**Primer sequences (5' – 3')**
GSTe1(E1)	Intergenic F Intergenic R	GGT GGA CTG TTG GGT TTC GTG GCA GGG TGG GCT CAG ATG ACC GG
GSTe2 (E2)	Arab E2 F Arab E2 R	TAT GTT GGC GAA TGG AA GGC TAA GGT GCA GGG TGT A

5.2.2.4 Alignment of sequencing data

Sequences were edited and built into relative contigs using Editseq and Seqman programmes from DNA Star software. Consensus sequences of promoter regions obtained in MAT and KGB strains and the field samples were aligned using Clustal W (Thompson *et al*, 1994).

5.2.2.5 Database sequence analysis

Initially, sequences were searched manually using the arthropod typical consensus sequence (TCAGT) to identify the initiator site and the core promoters in position at expected distances estimated from the initiator (Kadonaga, 2002). Subsequently, promoter sequences of *GSTe1* and *GSTe2* from the laboratory and field samples of *An. arabiensis* were analysed in more detail with the MatInspector programme (http://www.genomatrixide) to identify putative promoter elements and transcription factor binding sites. The programme is an on-line tool that utilises a library of matrix descriptions for transcription factor binding sites. Mat Inspector was used in preference to similar other software because its library (Matrix Family Library version 4.1) contains weighted matrix grouped into six, including fungi, plants, insects and vertebrates. It also contains other functional elements such as poly A signals and allows access to matrices from all databases (Quandt *et al*, 1995). The *GSTe1* and *GSTe2* promoters were analysed by setting the matrix library as insect, the core-similarity at 1.0 and matrix similarity at 0.9.

5.3 Results

5.3.1 Inducible expression of Epsilon *GSTe2* by DDT, permethrin and hydrogen peroxide.

Epsilon class GSTs have been implicated in pyrethroid and H_2O_2 induced oxidative stress and *GSTe2* has been specifically demonstrated to detoxify DDT (Vontas *et al*, 2001; Ranson *et al*,. 2001; Ortelli *et al,* 2003).Quantitative PCR was used to compare the relative abundance of the transcript of *GSTe2* in unexposed and larvae exposed to sub-lethal doses of the inducing compounds in parallel experiments.

GSTe2 expression levels were found to follow a very positively skewed (log-Normal) distribution, so were transformed to natural logarithms for statistical analysis. The geometric (detransformed log) means of the observations were analysed using one-way analyses of variances. Differences between different exposure conditions were compared for each treatment separately.The Tukey "honestly significant difference" multiple comparison test was used to investigate significant differences in *GSTe2* transcripts between strains and exposure conditions. Statistical significance was set at the conventional 5% level for all tests.

The geometric means levels of *GSTe2* expressed at 0, 1, 2 and 24 hours post-exposure to 0.05mg/l DDT for one hour are shown individually in Figure 5.1. At 1 hour post-exposure to DDT, the mean expression of *GSTe2* was raised relative to the control larvae by just over one half (57%) MAT strain ($p = 0.025$) but was more than doubled (108% increase) in the DDT resistant KGB strain ($p < 0.001$). At 2-hours, the mean *GSTe2* expression was now 60% lower than in the control larvae in the MAT strain ($p < 0.001$); in the KGB strain, however the mean gene expression remained numerically higher than control (by 29% i.e. by just over one quarter), this difference was not statistically significant ($p = 0.146$); At 24 hours , mean gene expression was 79% lower than in the control larvae in the MAT strain ($p < 0.001$); in the KGB strain, mean gene expression was lower than control by just 16% - this difference was not statistically significant ($p = 0.445$).

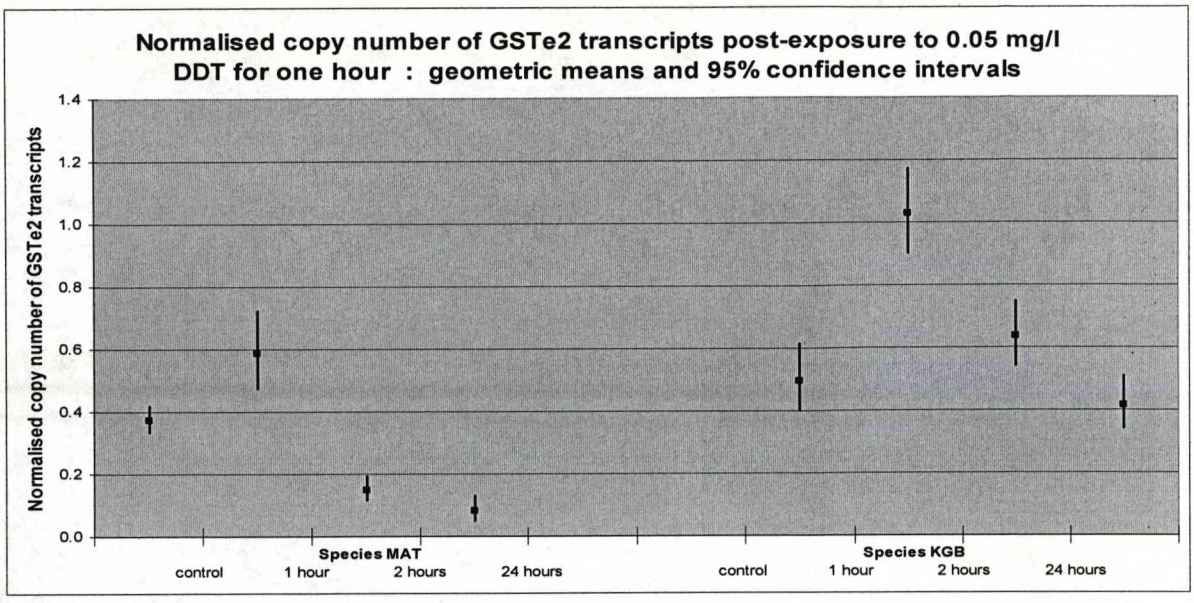


Figure 5.1: Induction of Epsilon *GSTe2* expression at 0, 1, 2 and 24 hours post-exposure to 0.05mg/L DDT for one hour – geometric means and 95% confidence intervals. Statistical differences in geometric means of the observations were calculated using Turkey honestly significant multiple tests.

For permethrin, the geometric means and the 95% confidence intervals for the observed levels of *GSTe2* expressed at 0, 2, 5 and 24 hours post-exposure to a sub-lethal dose of 0.02 mg/l permethrin for one hour are shown in Figure 5.2. A different pattern of expression of *GSTe2* was observed when larvae were exposed to permethrin.

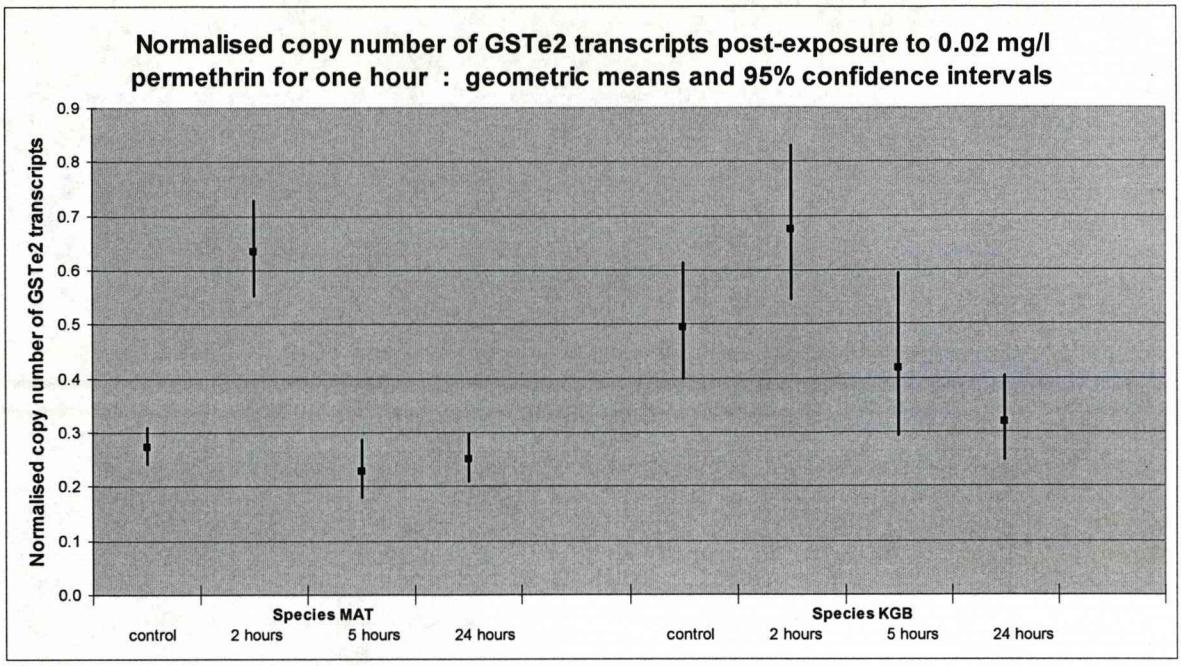


Figure 5.2: Induction of Epsilon *GSTe2* expression at 0, 2, 5 and 24 hours post-exposure to 0.02mg/L permethrin for one hour – geometric means and 95% confidence intervals.

The mean *GSTe2* expression peaked at 2 hours post-exposure, increasing by 132% in the MAT strain and by 146% in the KGB strain ($p < 0.001$ for both strains).The gene expression was 17% and 15.6% lower in the MAT and the KGB strains respectively at 5-hours post-exposure; this difference relative to the control strain was statistically not significant for the KGB strain ($p = 0.360$) and for the MAT strain ($p = 0.350$). At 24 hours post-exposure, mean *GSTe2* expression was 9% lower than control in the MAT strain but 16% higher in the KGB strain; however, neither difference was statistically significant ($p = 0.846$ for MAT and $p = 0.700$ for KGB).

The geometric means and their 95% confidence intervals for observed levels of *GSTe2* expressed at 0 and 1 hour post-exposure to H_2O_2 are shown individually in Figure 5.3.

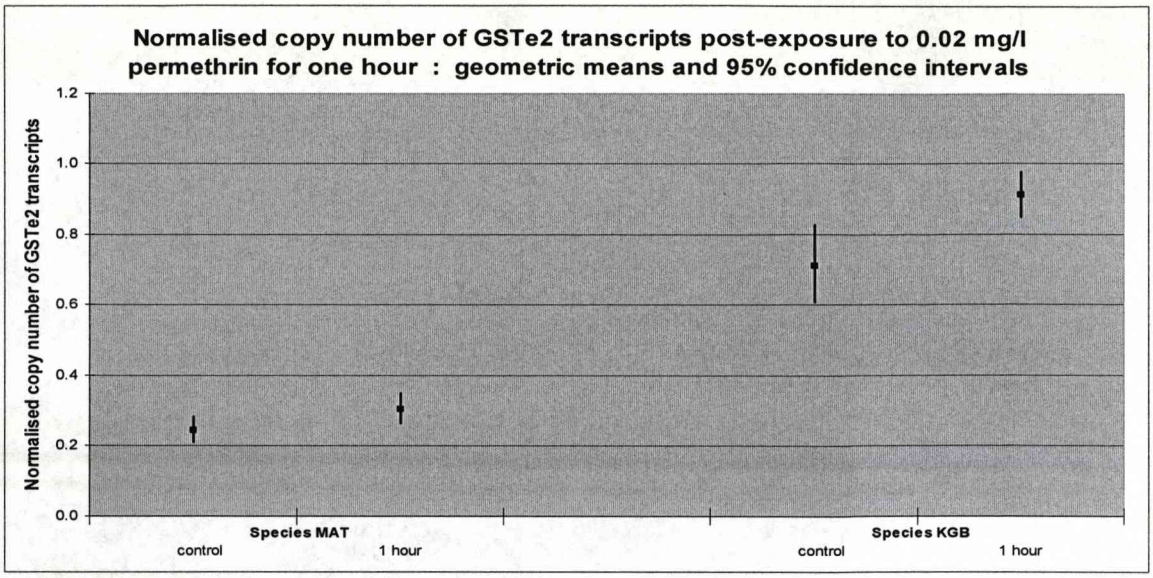


Figure 5.3: Induction of Epsilon *GSTe2* expression at 0 and 1 hours post-exposure to H_2O_2 – geometric means and 95% confidence intervals.

In contrast to DDT and permethrin, H_2O_2 produced only around a 25% increase in the expression of *GSTe2* at 1 hour post-exposure. However, the increases in the MAT strain (24%) and the KGB strain (28%) were both statistically significant ($p = 0.029$ and $p = 0.005$ respectively). The relative induced expression of *GSTe2* in different conditions is summarized in Table 5.2

Table 5.2: (Geometric) Mean *GSTe2* expression levels (with 95% confidence limits)

Species	Treatment	Time (hours)	Geometric Mean	(95% CI)
MAT	DDT	0	0.373	(0.335 – 0.416)
		1	0.587	(0.475 – 0.724)
		2	0.148	(0.115 – 0.191)
		24	0.080	(0.050 -.0.128)
MAT	PERMETHRIN	0	0.274	(0.242 – 0.309)
		2	0.634	(0.552 – 0.729)
		5	0.227	(0.180 -.0.287)
		24	0.250	(0.210 -.0.298)
MAT	H_2O_2	0	0.244	(0.210 – 0.284)
		1	0.303	(0.264 – 0.348)
KGB	DDT	0	0.494	(0.399 – 0.613)
		1	1.028	(0.901 – 1.172)
		2	0.636	(0.542 – 0.747)
		24	0.416	(0.340 – 0.509)
KGB	PERMETHRIN	0	0.494	(0.249 – 0.404)
		2	0.674	(0.546 – 0.831)
		5	0.417	(0.293 – 0.593)
		24	0.317	(0.249 – 0.404)
KGB	H_2O_2	0	0.710	(0.609 – 0.828)
		1	0.910	(0.848 – 0.977)

5.3.2 Comparison of the promoter sequences from laboratory strains and field samples of *An. arabiensis.*

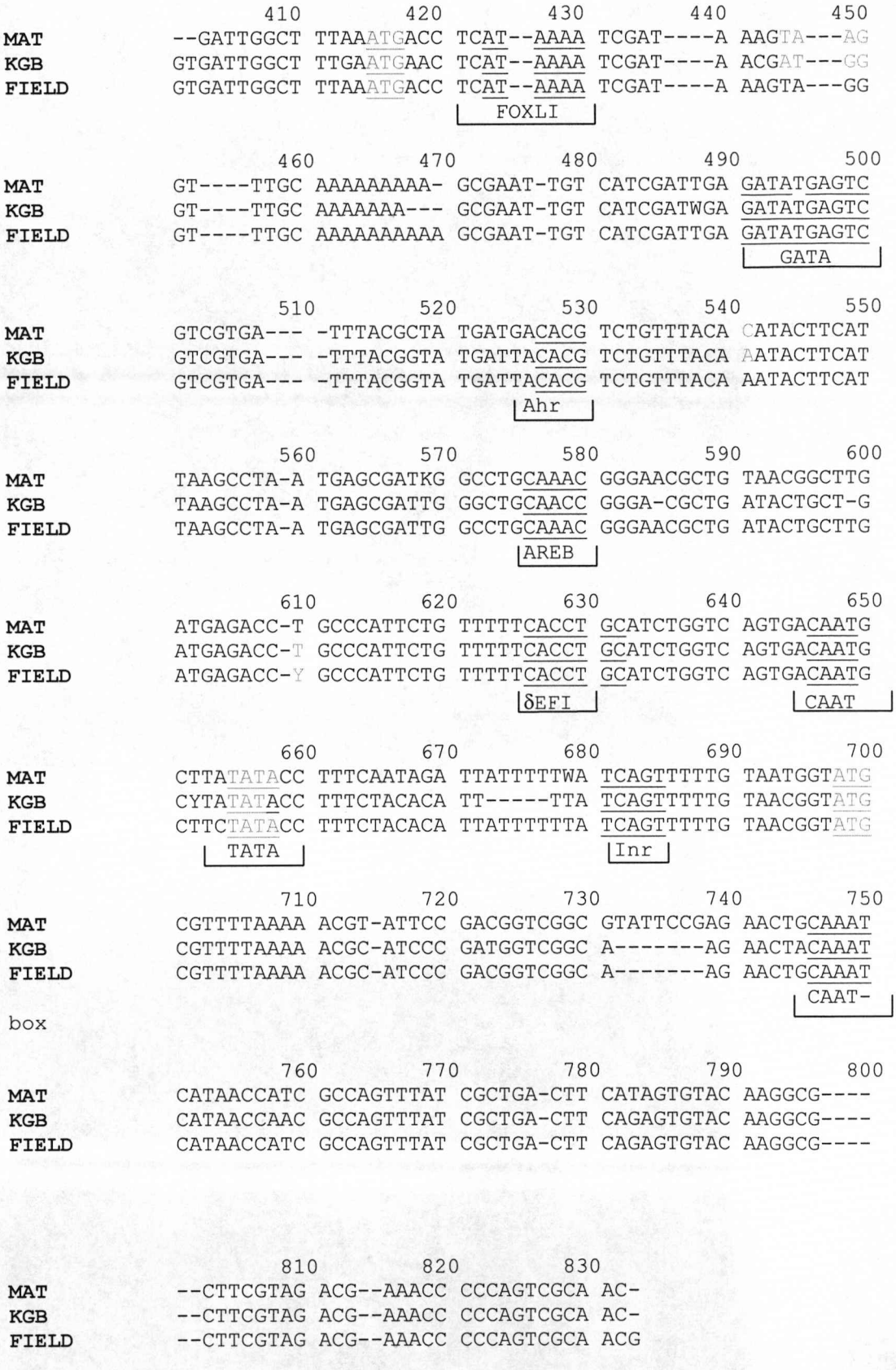
5.3.2.1 Sequencing the promoter region of *GSTe1*

Primers designed for homologous region of *An. gambiae* were used to amplify the intergenic space between *GSTe1* and *GSTe7* using genomic DNA from adult mosquitoes that were randomly sampled from laboratory colonies and field specimens of *An. arabiensis*. Three replicate genomic DNA samples each from 4 homogenized individuals from the MAT, KGB and field specimens were used as templates in the PCR so that individual variation of polymorphism and allelic changes between strains could be differentiated. Plasmids containing inserts of expected sizes were sequenced in both the forward and reverse orientation. A minimum of six clones were sequenced for each strain. The full-length sequences of the intergenic regions from the DDT-

resistant KGB, and susceptible MAT strains as well as the field samples were analysed both manually and using MatInspector to search for potential transcription factor binding sites (Figure 5.4). Analysis of the 832bp fragment revealed presence of core promoter elements including three putative transcription initiation sites consensus TCAGT (positions 685, 391, 10) and a CAAT BOX (positions 650) The first initiation site was located 13bp upstream of ATG translation start codon (Figure 5.4). No TATA box was found close to the third initiation site but another CAAT box was identified 27bp upstream close to the start codon. A further search using available software revealed other potential transcription factor binding sites of high homology (100% core similarity). Several other transcription factors identified in the *GSTe1* promoter were: Nuclear factor – kappa (NF – kb), Forkhead (FOXLI), Aryl hydrocarbon receptor (AhR) and GATA box (Figure 5.4).

Figure 5.4: Alignment of 806 bp region upstream of *GSTe1* in the Epsilon GST cluster in MAT and KGB strains as well as field samples of *An. arabiensis* using clustral W.

```
                    10         20         30         40         50
MAT       CGTTCAGCCA GAAACGCGTC ACGAACGTAT CCAAATGATG ATCTAACGTG
KGB       CGTTCAGTCT GAAACGCGTC ACGAACGTAT CCAAATGATG ATCCAACGTG
FIELD     CGTTCAGTCT GAAACGCGTC ACGAACGTAT CCAAATGATG ATCCAACGTG
            Inr                            CAAT

                    60         70         80         90        100
MAT       GACTAAGTTG AG-TCTACTT GTGCTACGTG CATCTCCGCA AAGAGAGATC
KGB       GACTAAGTTG AG-TCTACTT GTGCTACGTG CAGCTCCGCA AAGAGAGATC
FIELD     GACTAAGTTG AG-TCTACTT GTGCTACGTG CAGCTCCGCA AAGAGAGAT

                   110        120        130        140        150
MAT       ACCAAGTCGC GGGATAATAT CTTCCAAGAG CAACGACGAT GCAT------
KGB       ACCAAGTCGC GGGATAATAT CTTCCAAGAG CAACGACGAT GCAT------
FIELD     ACCWRGTCGC GGAATAATAT CTTCCAAGAG CGG-GATAAT ATCTTCCMAG
                      GATA

                   160        170        180        190        200
MAT       -GGCACTGAT AAGACGATTA GGGAACGGAA GAACTATCTA GGAGCGCTGG
KGB       -GGCACTGAT AAGACGATTA GGGAACGGAA GAACTATCTA GGAGCGCTGG
FIELD     AGGCACTGAT AAGACGATTA GGGAACSGAG GRATTATCTA GGAGCGCTGG

                   210        220        230        240        250
MAT       TTGGGATTGC TTTGGAAGTG ATAACATGAA GTAGCTACAA CTCTACTGGT
KGB       TTGGGATTGC TTTGGAAGAG ATWACATGAA GTAGCTACAA CTCTACTGGT
FIELD     TTGGGATTGG TTTGGAAGTG ATAACATGAA GTAGCTACAA CTCTACTGGT

                   260        270        280        290        300
MAT       GGTTACTCTT ATCTAGATGA CTGGG----- ---------- ---------A
KGB       GGTTACTCTT ATCTAGATGA CTGGGCTAGA TGACTTATCT AGATGTGGGA
FIELD     GGTTACTCTT ATCTAGATGA CTGGG----- ---------- ---------A

                   310        320        330        340        350
MAT       ATCTGCCCTA CTAGATTGCT GAAGTGTAGA GGGTTCTCWA CATCT--TTT
KGB       ATCTGCCCTA CTAGATTGCT AAAGTGTAGA TGGTTCTCAA CATCT--TTC
FIELD     ATCTGCCCTA CTAGATTGCT AAAGTGTAGA GGGTTCTCAA CATCT--TTT
            GC box

                   360        370        380        390        400
MAT       CTTAAT---- -ACACTTTCA TTTGAATAGA GAAATGACCT CAGT------
KGB       CTTAAT---- -ACACTTTCG TTTGAATAGC GAAAAGACCT CAGTACCTCA
FIELD     CTTAAT---- -ACACTTTCA TTTGAATAGC GAAATGACCT CAGTACCTCA
            TATA                                        Inr
```


410 420 430 440 450
MAT --GATTGGCT TTAAATGACC TCAT--AAAA TCGAT----A AAGTA---AG
KGB GTGATTGGCT TTGAATGAAC TCAT--AAAA TCGAT----A ACGAT---GG
FIELD GTGATTGGCT TTAAATGACC TCAT--AAAA TCGAT----A AAGTA---GG
FOXLI
460 470 480 490 500
MAT GT----TTGC AAAAAAAAA- GCGAAT-TGT CATCGATTGA GATATGAGTC
KGB GT----TTGC AAAAAAA--- GCGAAT-TGT CATCGATWGA GATATGAGTC
FIELD GT----TTGC AAAAAAAAAA GCGAAT-TGT CATCGATTGA GATATGAGTC
GATA
510 520 530 540 550
MAT GTCGTGA--- -TTTACGCTA TGATGACACG TCTGTTTACA CATACTTCAT
KGB GTCGTGA--- -TTTACGGTA TGATTACACG TCTGTTTACA AATACTTCAT
FIELD GTCGTGA--- -TTTACGGTA TGATTACACG TCTGTTTACA AATACTTCAT
Ahr
560 570 580 590 600
MAT TAAGCCTA-A TGAGCGATKG GCCTGCAAAC GGGAACGCTG TAACGGCTTG
KGB TAAGCCTA-A TGAGCGATTG GGCTGCAACC GGGA-CGCTG ATACTGCT-G
FIELD TAAGCCTA-A TGAGCGATTG GCCTGCAAAC GGGAACGCTG ATACTGCTTG
AREB
610 620 630 640 650
MAT ATGAGACC-T GCCCATTCTG TTTTTCACCT GCATCTGGTC AGTGACAATG
KGB ATGAGACC-T GCCCATTCTG TTTTTCACCT GCATCTGGTC AGTGACAATG
FIELD ATGAGACC-Y GCCCATTCTG TTTTTCACCT GCATCTGGTC AGTGACAATG
δEFI
CAAT
660 670 680 690 700
MAT CTTATATACC TTTCAATAGA TTATTTTTWA TCAGTTTTTG TAATGGTATG
KGB CYTATATACC TTTCTACACA TT-----TTA TCAGTTTTTG TAACGGTATG
FIELD CTTCTATACC TTTCTACACA TTATTTTTTA TCAGTTTTTG TAACGGTATG
TATA
Inr
710 720 730 740 750
MAT CGTTTTAAAA ACGT-ATTCC GACGGTCGGC GTATTCCGAG AACTGCAAAT
KGB CGTTTTAAAA ACGC-ATCCC GATGGTCGGC A-------AG AACTACAAAT
FIELD CGTTTTAAAA ACGC-ATCCC GACGGTCGGC A-------AG AACTGCAAAT
CAAT-
box
760 770 780 790 800
MAT CATAACCATC GCCAGTTTAT CGCTGA-CTT CATAGTGTAC AAGGCG----
KGB CATAACCAAC GCCAGTTTAT CGCTGA-CTT CAGAGTGTAC AAGGCG----
FIELD CATAACCATC GCCAGTTTAT CGCTGA-CTT CAGAGTGTAC AAGGCG----
810 820 830
MAT --CTTCGTAG ACG--AAACC CCCAGTCGCA AC-
KGB --CTTCGTAG ACG--AAACC CCCAGTCGCA AC-
FIELD --CTTCGTAG ACG--AAACC CCCAGTCGCA ACG

Potential core promoter elements including TATA,CAAT,GC boxes and an arthropod initiator are underlined and boxed. Other putative transcription factors and consensus sequences of high homology (100% core similarity and 95% matrix similarity)are underlined. Abbreviations: Nuclear factor – kabbab (nf – KB),Forkhead BOX (FOX LI),Aryl hydrocarbon receptor (AhR),GATA box are underlined. Allelic variations in sequences in this region between DDT resistant KGB and susceptible MAT strains as well as field samples are shaded in grey.

In contrast to the situation in *An. arabiensis*, the *GSTe1* intergenic region in both Zanu and Kisumu strains of *An. gambiae* lack the TATA box and other potential transcription factor biding sites including FOX LI, AREB, CAAT and δEFI (Figure 5.5). The arthropod initiator element at position 680 bp common to all strains is underlined and the start codon boxed. The core promoter, TATA and other potential transcription factors not found in *An. gambiae* are underlined, shaded in gray and boxed (Figure 5.5).

Figure 5.5: Comparison of the *GSTe1* intergenic regions in ZANU and KISUMU strains of *An. gambiae* to MAT , KGB and Field strains of *An. arabiensis* .

```
                  10         20         30         40         50
ZANU      -TCTAAGTCA GCGATAAACT GGCGATGGTT ATGATTTGCA GTTCT-TGCC
KISUMU    -TCTAAGTCA GCGATAAACT GGCGATGGTT ATGATTTGCA GTTCT-TGCC
MAT       CGTTCAGCCA GAAACGCGTC ACGAACGTAT CCAAATGATG ATCTAACGTG
KGB       CGTTCAGTCT GAAACGCGTC ACGAACGTAT CCAAATGATG ATCCAACGTG
FIELD     CGTTCAGTCT GAAACGCGTC ACGAACGTAT CCAAATGATG ATCCAACGTG
          INITIATOR

                  60         70         80         90         100
ZANU      GACC--GTTG GGATGCGTTT TTAAAACGCA TACCGTTACA AAAACTGATA
KISUMU    GACC--GTCG GGATGCGTTT TTAAAACGCA TACCGTTACA AAAACTGATA
MAT       GACTAAGTTG AG-TCTACTT GTGCTACGTG CATCTCCGCA AAGAGAGATC
KGB       GACTAAGTTG AG-TCTACTT GTGCTACGTG CAGCTCCGCA AAGAGAGATC
FIELD     GACTAAGTTG AG-TCTACTT GTGCTACGTG CAGCTCCGCA AAGAGAGATC

                  110        120        130        140        150
ZANU      A--------- AAAATAATGT GT----AGAA AGGTATAGAA GCATT-----
KISUMU    A--------- AAAATAATGT GT----AGAA AGGTATAGAA GCATT-----
MAT       ACCAAGTCGC GGGATAATAT CTTCCAAGAG CAACGACGAT GCAT------
KGB       ACCAAGTCGC GGGATAATAT CTTCCAAGAG CAACGACGAT GCAT------
FIELD     ACCWRGTCGC GGAATAATAT CTTCCAAGAG CGG-GATAAT ATCTTCCMAG
                     GATA
```

```
                    160        170        180        190        200

ZANU        -GTCATTGAC CAGATG--CA GGT----GAA AAAC-----A GAA----TGG
KISUMU      -GTCACTGAC CAGATG--CA GGT----GAA AAAC-----A GAA----TGG
MAT         -GGCACTGAT AAGACGATTA GGGAACGGAA GAACTATCTA GGAGCGCTGG
KGB         -GGCACTGAT AAGACGATTA GGGAACGGAA GAACTATCTA GGAGCGCTGG
FIELD       AGGCACTGAT AAGACGATTA GGGAACSGAG GRATTATCTA GGAGCGCTGG

                  210        220        230        240        250
ZANU        GCAGGTCTCA TCAAGCAGTA TCAGCGTTCC -CGTTTGCAG GCCAATCGCT
KISUMU      GCAGGTCTCA TCAAGCAGTA TCAGCGTTCC -CGTTTGCAG GCCAATCGCT
MAT         TTGGGATTGC TTTGGAAGTG ATAACATGAA GTAGCTACAA CTCTACTGGT
KGB         TTGGGATTGC TTTGGAAGAG ATWACATGAA GTAGCTACAA CTCTACTGGT
FIELD       TTGGGATTGG TTTGGAAGTG ATAACATGAA GTAGCTACAA CTCTACTGGT

                  260        270        280        290        300
ZANU        CATTAGGCTT AATGAAGTAT TTGT------ ---------- ---------A
KISUMU      CATTAGGCTT AATGAAGTAT TTGT------ ---------- ---------A
MAT         GGTTACTCTT ATCTAGATGA CTGGG----- ---------- ---------A
KGB         GGTTACTCTT ATCTAGATGA CTGGGCTAGA TGACTTATCT AGATGTGGGA
FIELD       GGTTACTCTT ATCTAGATGA CTGGG----- ---------- ---------A

                  310        320        330        340        350
ZANU        AACAGACGTG T--AATCAT- --AGCGTAAA ----TCACGA CGACTCATAT
KISUMU      AACAGACGTG T--AATCAT- --ACCGTAAA ----TCACGA CGACTCATAT
MAT         ATCTGCCCTA CTAGATTGCT GAAGTGTAGA GGGTTCTCWA CATCT--TTT
KGB         ATCTGCCCTA CTAGATTGCT AAAGTGTAGA TGGTTCTCAA CATCT--TTC
FIELD       ATCTGCCCTA CTAGATTGCT AAAGTGTAGA GGGTTCTCAA CATCT--TTT

                  360        370        380        390        400
ZANU        CTCAATCGAT GACAATTCGC TTTTTT---- GCAA--ACCC TACT------
KISUMU      CTCAATCGAT GACAATTCGC TTTTTTTTTT GCAA--ACCC TACT------
MAT         CTTAAT---- -ACACTTTCA TTTGAATAGA GAAATGACCT CAGT------
KGB         CTTAAT---- -ACACTTTCG TTTGAATAGC GAAAAGACCT CAGTACCTCA
FIELD       CTTAAT---- -ACACTTTCA TTTGAATAGC GAAATGACCT CAGTACCTCA
                TATA                                    INITIATOR

                  410        420        430        440        450
ZANU        -TTATCGATT TT--ATGAGG TCATTTAAAG CCAATCACTG AGGTACTGAG
KISUMU      -TTATCGATT TT--ATGAGG TCATTTAAAG CCAATCACTG AGGTACTGAG
MAT         --GATTGGCT TTAAATGACC TCAT--AAAA TCGAT----A AAGTA---AG
KGB         GTGATTGGCT TTGAATGAAC TCAT--AAAA TCGAT----A ACGAT---GG
FIELD       GTGATTGGCT TTAAATGACC TCAT--AAAA TCGAT----A AAGTA---GG

                  460        470        480        490        500
ZANU        GTCTTTTCGC TATTCAA--- ACGAAAGTGT -----ATTAA GTAA--AGAT
KISUMU      GTCATTTCGC TATTCAA--- ATGAAAGTGT -----ATTAA GAAA--AGAT
MAT         GT----TTGC AAAAAAAAA- GCGAAT-TGT CATCGATTGA GATATGAGTC
KGB         GT----TTGC AAAAAAA--- GCGAAT-TGT CATCGATWGA GATATGAGTC
FIELD       GT----TTGC AAAAAAAAAA GCGAAT-TGT CATCGATTGA GATATGAGTC
                FOX LI SITE
```

```
                  510         520         530         540         550
ZANU        GTTGAGAACC CTCTACACTT TAGCAATCAG TAGG---GCA GATTCCTAGT
KISUMU      GTTGAGAACC CTCTACACTT TAGCAATCAG TAGG---GCA GATTCCCAGT
MAT         GTCGTGA--- -TTTACGCTA TGATGACACG TCTGTTTACA CATACTTCAT
KGB         GTCGTGA--- -TTTACGGTA TGATTACACG TCTGTTTACA AATACTTCAT
FIELD       GTCGTGA--- -TTTACGGTA TGATTACACG TCTGTTTACA AATACTTCAT

                  560         570         580         590         600
ZANU        CA--TCTAGA TAAGAG--TA ACC----ACC AGTAGAGTTG TAGCTACTTC
KISUMU      CA--TCTAGA TAAGAG--TA ACC----ACC AGTAGAGTTG TAGCTACTTC
MAT         TAAGCCTA-A TGAGCGATKG GCCTGCAAAC GGGAACGCTG TAACGGCTTG
KGB         TAAGCCTA-A TGAGCGATTG GGCTGCAACC GGGA-CGCTG ATACTGCT-G
FIELD       TAAGCCTA-A TGAGCGATTG GCCTGCAAAC GGGAACGCTG ATACTGCTTG
                  610         620                     640         650
                                        AREB
ZANU        ATGTTATCAC TTCCAAAGCA ATCCCAACCA GC----GCTC CTAGATAGTT
KISUMU      ATGTTATCAC TTCCAAACCA ATCCCAACCA GC----GCTC CTAGATAATT
MAT         ATGAGACC-T GCCCATTCTG TTTTTCACCT GCATCTGGTC AGTGACAATG
KGB         ATGAGACC-T GCCCATTCTG TTTTTCACCT GCATCTGGTC AGTGACAATG
FIELD       ATGAGACC-Y GCCCATTCTG TTTTTCACCT GCATCTGGTC AGTGACAATG
                                    δEF1                    CAAT

                  660         670         680         690         700
ZANU        CTTCCGTTCC ---CTAATCG TC-----TTA TCAGTGCATG CATCGTCGTG
KISUMU      CCTCCGTTCC ---CTAATCG TC-----TTA TCAGTGCATG CATCTTCGTG
MAT         CTTATATACC TTTCAATAGA TTATTTTTWA TCAGTTTTTG TAATGGTATG
KGB         CYTATATACC TTTCTACACA TT-----TTA TCAGTTTTTG TAACGGTATG
FIELD       CTTCTATACC TTTCTACACA TTATTTTTTA TCAGTTTTTG TAACGGTATG
              TATA                                        S. CODON

                  710         720         730         740         750
ZANU        CTCTTGGAAG ATATTATCCC GCGACTTGGT G--------- ----------
KISUMU      CTCTTGGAAG ATATTATCCC GCGACTTGGT G--------- ----------
MAT         CGTTTTAAAA ACGT-ATTCC GACGGTCGGC GTATTCCGAG AACTGCAAAT
KGB         CGTTTTAAAA ACGC-ATCCC GATGGTCGGC A-------AG AACTACAAAT
FIELD       CGTTTTAAAA ACGC-ATCCC GACGGTCGGC A-------AG AACTGCAAAT

                  760         770         780         790         800
ZANU        -------ATC TC----TCTT TGCGGAGCTG CACGTAGCAC AAGTAGACTC
KISUMU      -------ATC TC----TCTT TGCGGAGCTG CACGTAGCAC AAGTAGACTC
MAT         CATAACCATC GCCAGTTTAT CGCTGA-CTT CATAGTGTAC AAGGCG----
KGB         CATAACCAAC GCCAGTTTAT CGCTGA-CTT CAGAGTGTAC AAGGCG----
FIELD       CATAACCATC GCCAGTTTAT CGCTGA-CTT CAGAGTGTAC AAGGCG----

                  810         820         830
ZANU        AACTTAGTCC ACGTTAGATC ATCATTTG-- ---
KISUMU      AACTTAGTCC ACGTTAGATC ATCATTTG-- ---
MAT         --CTTCGTAG ACG--AAACC CCCAGTCGCA AC-
KGB         --CTTCGTAG ACG--AAACC CCCAGTCGCA AC-
FIELD       --CTTCGTAG ACG--AAACC CCCAGTCGCA ACG
```

5.3.3.4 Sequencing the promoter region of *GSTe2*

On assessment of the alignment of the *GSTe2* promoter sequences in *An. arabiensis*, several potential transcription factors and regulatory binding sites were identified including a typical arthropod initiator consensus, T-C-A-G/T-T-T/C (Purnell et al.; 1994), a GC box and a CAAT box (Figure 5.6). No TATA box was identified in the *GSTe2* promoter. In addition, a number of motifs for GATA box, Fox LI, AhR, NF-kb and AREB were identified in the putative *GSTe2* promoter (Figure 5.6).

Figure 5.6: Comparison of the sequences of the intergenic region of *GSTe1* and *GSTe2* between *An. arabiensis* MAT, KGB and field strains and those of *An. gambiae* ZAN/U and Kisumu strains.

```
                10          20          30          40          50
MAT             TATGTTGGCG AAGAATGCAA AACTGTAACA GGT-TCAAGC TATTCGCGGT
KGB             TATGTTGGCG AAGAATGCAA AACTGTAACA GGT-TCAAGC TATTCGCGGT
FIELD           TATGTTGGCG AAGAATGCAA AACTGTAACA GGT-TCAAGC TATTCGCGGT
ZANU            ---------- ---------- --------CA GGTACCGTGC TATTCGCGGT
KISUMU          ---------- ---------- --------CA GGTACC-TGC TATTCGCGGT

                60          70          80          90         100
MAT             AGTTAACTAG TCAAATAAAG TGTAGAATTT AAAGCACCCA GTAACGTGTT
KGB             AGTTAACTAG TCAAATAAAG TGTAGAATTT AAAGCACCMA GTAACGTGTT
FIELD           AGTTAACTAG TCAAATAAAG TGTAGAATTT AAAGCACCMA GTAACGTGTT
ZANU            TGTTAACTAG TCAAATAAAG TGTAGGATTT TAAGCACCTA GTTACGTTTT
KISUMU          TGTTAACTAG TCAAATAAAG TGTAGGATTG AAAGCACCCA GTAACGTTTT

                110         120         130         140         150
MAT             TCTTGTGCAT AAAAAAA-CA GGAATTCGCT TCTGCTTTTA T--GTTGCAG
KGB             TCTTGTGCAT AAAAAA--CA GGAATTCGCT TCTGCTTTTA T--GTTGCAG
FIELD           TCTTGTGCAT AAAAAAA-CA GGAATTCGCT TCTGCTTTTA T--GTTGCAG
ZANU            TCTTGTGCAT AAAAAA--CA GGAATTCGCT TCTGCTTTAA AAGATTGCAG
KISUMU          TCTTGTGCAT AAAAAAAACA GGAATTCGCT TCTGCTTTTA T--GTTGCAG
                          FOXL1

                160         170         180         190         200
MAT             TACGAAGAAC CGG-AATGAG TATG-AAATA AATTCGCCGA CACGCACACA
KGB             TACGAAGAAC CGG-AATGAG TATG-AAATA AATTCGCCGA CACGCACACA
FIELD           TACGAAGAAC CGG-AATGAG TATG-AAATA AATTCGCCGA CACGCACACA
ZANU            TACGAAGAAC CGGTAATGAG TATGTAAATA AATTCGCCGA CACGCACACA
KISUMU          TACGAAGAAC CGG-AATGAG TATG-AAATA AATTCGCCGA CACGCACACA
                                                          AhR

            210         220         230         240         250
MAT           -ACAACACCA ACCCCAAGTA ATGCTTTATC TCGGGCCCTT CCAACGAACA
KGB           -ACAACACCA ACCCCAAGTA ATGCTTTATS TCGGGCCCTT CCAACGAACA
FIELD         -ACAACACCA ACCCCAAGTA ATGCTTTATG TCGGGCCCTT CCAACGAACA
ZANU          TAAAACACCA ACCCCTAGTA ATGCTT-ATC TCGGGCCCTT CCTACGTATA
KISUMU        -ACAACACCA ACCCCAAGTA ATGCTTTATC TCGGGCCCTT CCAACGAACA
                                  GATA          NF-kb
```

```
                210         220         230         240         250
MAT               -ACAACACCA ACCCCAAGTA ATGCTTTATC TCGGGCCCTT CCAACGAACA
KGB               -ACAACACCA ACCCCAAGTA ATGCTTTATS TCGGGCCCTT CCAACGAACA
FIELD             -ACAACACCA ACCCCAAGTA ATGCTTTATG TCGGGCCCTT CCAACGAACA
ZANU              TAAAACACCA ACCCCTAGTA ATGCTT-ATC TCGGGCCCTT CCTACGTATA
KISUMU            -ACAACACCA ACCCCAAGTA ATGCTTTATC TCGGGCCCTT CCAACGAACA
                                        └── GATA ──┘ └─ NF-kb ─┘

                260         270         280         290         300
MAT               CAGTCGCACA TCCGAAGCAT GGGGCGTTTG CTATGCTTGG GGTCGATGCT
KGB               CAGTCGCACA TCCGAAGCAT GGGGCGTTTG CTATGCTTGG GGTCGATGCT
FIELD             CAGTCGCACA TCCGAAGCAT GGGGCGTTTG CTATGCTTGG GGTCGATGCT
ZANU              CAATCACACA TCCGTAGCAT GGGGCGTTTG CTATGCTTGG GGTCGATGCT
KISUMU            CAGTCACACA TCCGAAGCAT GGGGCGTTTG CTATGCTTGG GGTCGATGCT
                  310         320         330         340         350
MAT               ACA-AACATC CACAGCCAAT CATCAGTTCG GGTGTGTCTC TTG-AACGTG
KGB               ACA-RACATC CACAGCCAAT CATCAGTTSG GGTGTGTCTC TTG-AACGTG
FIELD             ACA-GACATC CACAGCCAAT CATCAGTTCG GGTGTGTCTC TTG-AACGTG
ZANU              ACATAACATC CACAGCCAAT CATCAGTTCG GGTGTGTCTC TTGTAACGTG
KISUMU            ACA-AACATC CACAGCCAAT CATCAGTTCG GGTGTGTCTC TAG-AACGTG
                             └─ CAAT ─┘ └─Inr ─┘

                360         370         380         390
MAT               TACGGTGTGT GTCCATCCAG TTGCGAAAAT GTCCAAGCTT GTACTGT
KGB               TACGGTGTGT GTCCATCCAG TTGCGAAAAT GTCCAAGCTT GTACTG-
FIELD             TACGGTGTGT GTCCATCCAG TTGCGAAAAT GTCCAAGCTT GTACTG-
ZANU              TACGGTGTGT GTCCATCCAG TTTTTA---- GTTC--GCTG CTAGC--
KISUMU            TACGGTTTGT GTCCATCCAG TTTTTA---- GTTC--GCTG CTAGC-
```

Core promoters (boxed) and transcriptional elements (underlined) which are identified in this region are indicated. Additional abbreviations are: nuclear matrix protein (NMP4), C – EST and C – Myb. Allelic variations within strains and between species are shaded in gray.

5.4 Discussion

5.4.1 Induction of *GSTe2* by DDT, permethrin and H_2O_2

In this study, the induction of *GSTe2* expression by the three xenobiotic compounds, DDT, permethrin and H_2O_2 was investigated. *An. arabiensis* Epsilon *GSTe2* was induced significantly, by approximately 50% in the MAT and KGB strains one hour post-exposure to DDT. This result is similar to that reported for induced GST activity by DDT in *Musca domestica* (Hayaoka and Dauterman, 1982). The expression of *GSTe2* decreased after two hours by approximately 42% in the DDT-treated compared to control larvae in MAT strain, but in the KGB strain, the expression remains induced though not significantly ($p = 0.146$) (Figure 5.2). This observed difference in the induction pattern of *GSTe2* suggests higher intrinsic levels of GST activity in the KGB compared to the MAT strain probably due to the difference in area of their origin. DDT was a more potent inducer of *GSTe2* than permethrin and H_2O_2 (Table

5.2). The stronger induction of *GSTe2* expression by DDT compared to permethrin and the H_2O_2 may be explained on the basis of substrate specificities.

A border line significant induction of *GSTe2* expression was observed (1.2-fold) in MAT and (1.4-fold) in KGB strains following exposure to 3 mM H_2O_2 (Table 5.2). This result is very similar to that reported for H_2O_2 induced expression of *GSTe2* at one hour time point in *An. gambiae* (Ding *et al.,* 2005). The expression of *GSTe2* was induced 1.7 and 1.2 fold in ZAN/U and Kisumu strains respectively. The induction of *GSTe2* expression by H_2O_2 in *An. gambiae* was suggested to be due to the presence of transcription factors responsive to oxidative stress in the promoter region of the gene. The induction of *GSTe2* expression by different chemical compounds may suggest its involvement in the detoxification of xenobiotic compounds found in the natural environment of the mosquito larvae. Elevated tolerance of mosquito larvae to chemical insecticides after their exposure to common herbicides has been demonstrated and this indicated the potential of pesticide residues in larval habitats to confer mosquito larvae pre-adaptive advantage to develop insecticide resistance (Boyer *et al.,* 2006). In this respect, a comparative study of molecular mechanisms controlling the induction of individual GST genes between *An. arabiensis* and *An. gambiae* may help to understand the ecological differences identified between these important malaria vectors (Coluzzi *et al.,* 1979).

5.4.2 Sequence analysis of *GSTe1* and *GSTe2* promoters

The typical regulatory DNA sequences (core promoters) and transcriptional elements often found in the promoter of a eukaryotic structural gene is schematically illustrated in Figure 5.7.

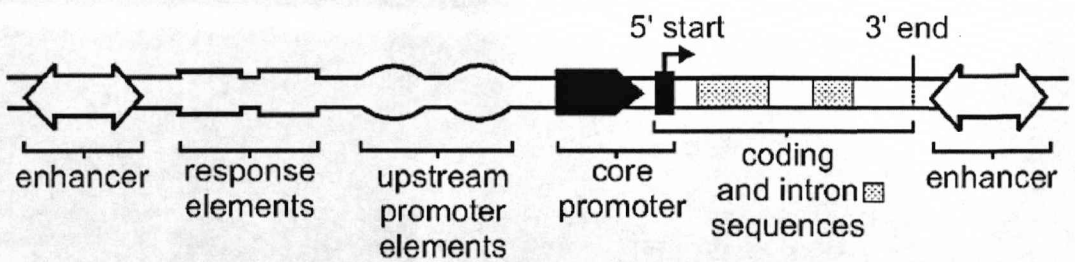


Figure 5.7: DNA sequences, associated with a gene, that act to regulate transcription.(reviewed by Harshman and James, 1998)

The core promoter includes a TATA box consensus (TATAAA), an initiator (TCAGT), a TF11B recognition element and a down stream element (Weaver and Hendrick, 1997). Regulatory sequences found proximal to the 5' end of the gene, that are collectively termed as upstream promoter elements (UPE) consist of conserve sequences such as CAAT, GC and OCT (Harshman and James, 1993).

Manual and computer analysis of the *GSTe1* promoter in DDT resistant KGB and susceptible MAT strains, as well as field samples, identified several core promoter elements, including a classic TATA box, 3 CAAT and 5 GC boxes (Figure 5.5). A typical TATA box was identified approximately 37 bp upstream of the TSS of *GSTe1* promoter in all the strains and the field specimens.

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In addition to the TATA box, the repetitive presence of 5 bp sequence TCAGT was noted, two of which were located at 12 bp and at 29 bp upstream of the TSS (Figure 5.5). This sequence is identical to the arthropod initiator consensus sequence, which is significant in transcription of many arthropod promoters (Cherbas and Cherbas, 1996). The simultaneous occurrence of these conserved regulatory sequences at the two extreme ends of this intergenic region is probably indicative of the opposite transcriptional orientations of *GSTe1* and the neighbouring *GSTe7* gene in the Epsilon class gene cluster (Ding *et al*, 2003). The *GSTe1* promoter in *An. arabiensis* posseses a TATA box and an arthropod initiator sequence, as has been reported for genes that are specifically regulated in mosquito fat bodies (Choi *et al,* 1997). In *Culex quinquefasciatus*, the functionality of the arthropod initiator sequence in transcriptional regulation of β-esterase gene has been demonstrated (Hawkes and Hemingway, 2002). A base substitution (T to C) was noted between the initiator sequence of the resistant KGB and susceptible MAT strain. The significance of the genetic differences in this intergenic region between *An. gambiae* and *An. arabiensis* and the transcriptional role of the initiator sequences in *An. arabiensis GSTe1* promoter needs further investigation.

Simultaneous analysis of *GSTe2* promoter region in *An. arabiensis* also revealed the presence of a potential initiator sequence, but no TATA box was found in the promoter region of the gene (Figure 5.6). In the absence of a TATA box, the initiator

functions to localise a transcription start site (TSS) and mediates the action of the upstream activators. Transcription carried out by an initiator is usually from a position that overlaps a TSS (Smale, 1997). A downstream promoter element (DPE) consensus (A/G-G-A/T-C/T-G/A/C) located +28 to 32 relative to the initiator, was mainly present in core promoters that lack a TATA box motif (Kutach and Kadonaga, 2000). However, no potential DPE element was identified downstream of the putative initiator in the *GSTe2* promoter in *An. arabiensis*. Similar investigations of the transcriptional control elements of *GSTe2* promoter in *An. gambiae* have identified an initiator and confirmed the absence of a TATA box and a DPE element motif (Ding *et al,* 2005). A two adenosine indel was identified in the *GSTe2* promoter in the DDT resistant KGB strain, but was not found in the susceptible MAT and field samples of *An. arabiensis* (Figure 5.6). The alignment of the *An. arabiensis GSTe2* promoter sequences from the three strains with sequences of *An. gambiae GSTe2* promoter from the DDT resistant ZAN/U and susceptible Kisumu strain, revealed a striking similarity of the regulatory regions between the strains. The AA-indel in the DDT resistant *An. arabiensis* KGB strain is identical to that in resistant ZAN/U *An. gambiae* (Figure 5.6). Ding *et al,* (2005) used site directed mutagenesis to demonstrate the functional role of the two adenosine indel in conferring elevated transcriptional activity in the *An. gambiae* resistant strain. Therefore, it is assumed that due to considerable conservation in sequence and position, the AA-indel although at a slightly different position in *An. arabiensis*, may have similar function to that in *An. gambiae*, but this needs further verification.

Sequences that are located between 50 bp and 200 bp upstream of the TSS containing motifs such as CCAAT, GC, OCT are referred to as upstream promoter elements UPE (Harshman and James, 1998). The GC box activates the promoter activity, whereas the CCAAT box binds to a variety of proteins, either enhancing or decreasing the basal transcription obtained by the core promoter. Sequences that correspond to several classes of upstream promoter elements are identified upstream of the *GSTe1* and *GSTe2* genes in both strains of *An. arabiensis* (Figures 5.5, 5.6).

The GC box interacts with SP1 and is essential for transcriptional initiation and positive regulation of transcription of several genes (Fry and Farnham,1999). Presence of similar upstream promoter sequences in *An. gambiae* Epsilon class *GSTe2* and *GSTe3* genes has been reported (Ding *et al,* 2005). However, the roles of the

CCAAT and GC boxes in the transcription of these genes have not been investigated in the two Anopheline species. The DNA sequences designated as environmental response element are speculated to be involved in the induction of gene expression in response to hormonal signals or environmental stimuli (Harshman and James, 1998). Several transcription factors responsive to oxidative stress, including Forkhead transcription factor Fox L1 (Burgering and Kops, 2002; Kops *et al,* 2002) and NF – kb (Schreck *et al,* 1992) and AhR (Haddad, 2004) were identified in the *GSTe1* and *GSTe2* promoters in both the resistant and susceptible strains of *An. arabiensis*. NF – Kb is an important transcription factor that responds directly to oxidative stress in eukaryotic cells and was shown to be activated by low concentrations of H_2O_2 (Schreck *et al.,* 1992). Activation of the Fox factor by oxidant treatment has been associated with the elevated expression of detoxification enzymes (Burgering and Kops, 2002; Kops *et al,* 2002).

In *An. gambiae* the presence of Fox L1, NF – Kb and AhR in *GSTe2* promoter was used to explain partially the induced expression of the gene by H_2O_2. In *An. arabiensis* the expression of *GSTe2* was increased by exposure of larvae from the KGB and MAT strains to DDT, permethrin and H_2O_2. The oxidative stress related transcription factors mentioned above, were identified unexclusively in both strains. However, functional studies need to be conducted to test the hypothesis of their involvement in gene induction. Also, it will be informative to test the effects of these xenobiotic compounds on induction of other members of Epsilon class GST genes in *An. arabiensis.*

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1 Selection for DDT resistance in the colonies studied.

The main objective of this study was to identify the biochemical and molecular mechanisms conferring resistance to DDT in the MAT and KGB laboratory strains of *An. arabiensis*. To achieve this goal it was necessary to select the colonies for DDT resistance and to perform comparative biochemical studies of the detoxification systems between the parental and DDT selected populations of each strain. As an essential first stage in the investigation, the initial susceptibility or resistance to DDT of the parental colonies was determined. The presence of low levels of the resistance genotypes segregating in the parental populations was noted. For example, in the MAT parental colony, the WHO standard susceptibility bioassay indicated a low level of resistance. This knowledge was used to establish the selecting dose that was applied each generation to the progeny of the parental population to select for the resistance genotypes. Tests result showed 87% mortality 24 hours post-exposure to 4% DDT for one hour, in successive generations. The patterns in the resistance levels in the different generations under DDT selection pressure in both the MAT and KGB strains is depicted by the percentage mortality data recorded by WHO susceptibility bioassays (Chapter 2). The fluctuation in mortality that was observed between generations during the period of selection indicates that the population was heterozygous with respect to DDT resistance.

In the present study, selection to, or near to, homozygosity with respect to the resistance gene either in the MAT or KGB strain, was not achieved. In fact RR of only 1.5× (MAT) and 2.2× (KGB) were obtained. This might partially be due to low intensity of the selection and the multifactorial nature of DDT resistance in the populations. The type of response to DDT selection shown by the MAT and KGB colonies is often explained in terms of interactions of numerous variable factors important among which include the number of genes involved, mode of inheritance, genetic variance, population size and intensity of selection (Rosenheim and Tabashnik, 1990; Georghiou and Taylor, 1977). Investigation into the mode of inheritance and analysis of interactions of these factors, with regards to DDT

selection in the colonies, was not attempted in the present study, but their consideration is pertinent to the discussion. Davidson (1958) was the first to demonstrate, using laboratory crossing experiments and WHO bioassays, that DDT resistance was completely recessive in *An. albimanus*, almost completely recessive in *An. quadrimaculatus* and more recessive than dominant in *An. stephensi*. He concluded that single alleles were responsible for DDT resistance in all three species. The genetics of DDT resistance has been studied in eight Anopheline species including *An. gambiae* and *An. arabiensis* (Haridi, 1970, 1972). Resistance in most cases is due to a single, incompletely dominant gene. However, even within a single species resistance may be dominant in one population and recessive in another. Ranson *et al,*(2000a), using a genetic mapping approach, have identified two major QTLs that were associated with DDT resistance in different regions of the genome of *An. gambiae*, suggesting multifactorial resistance. The extent of the genetic factors that are involved in DDT resistance in both the MAT and KGB strains remain to be investigated.

After selecting the colonies for physiological resistance to DDT, it was logical to determine whether selection with DDT increases the level of resistance to permethrin. The evidence of cross-resistance studies indicates that DDT selection has produced increased tolerance to permethrin in the MAT colony, but this phenomenon was not investigated in the selected population in the KGB strain due to low numbers. The population under DDT selection pressure showed significantly increased tolerance to early and late knock-down effects of permethrin than the parental population (Figure 2.5). This result is similar to the data reported for the G1 colony of *An. arabiensis* (Hemingway, 1981). Knock-down time has long been accepted as an indicator of susceptibility and its measurement provides initial information on the possible involvement of a kdr allele (Chandre *et al*, 1999; Kang, 1995).

The Cross resistance between DDT and pyrethroid appears to be correlated to the degree of DDT resistance. Plapp and Hoyer (1968) found that a DDT resistant strain (X66) of *Culex tarsalis* possessed cross-resistance of 6.5X to piperonyl butoxide synergised pyrethrins. They attributed this cross-resistance to a *kdr*-like mechanism. A highly DDT resistant field strain of *Aedes aegypti* (RR = 19-73X)

showed as much as 30X cross-resistance to pyrethroids (Prasittisuk and Busvine, 1977). Similarly, previous studies have shown evidence for the involvement of the DDT resistance mechanisms in pyrethroid resistance in mosquitoes. Biochemical, synergist and cross-resistance studies have implicated metabolic and *kdr*-like mechanisms in various DDT/pyrethroid resistant mosquitoes, including *An. sacharovi* (Herath et al. 1988), *An. stephensi* (Omer *et al,* 1980), *An. gambiae* (Vulule *et al,* 1994; Martinezz-Torres *et al,* 1998; Ranson *et al,* 2001) and *An. arabiensis* (Ratovonjatu et al. 2003).

6.2 The Extent and Characterisation of the An. arabiensis Epsilon GST class.

In this study eight Epsilon GST genes have been identified in *An. arabiensis*. Hitherto, there has been no previous report on the *An. arabiensis* GST gene family and the genome sequence of this important African malaria vector is not yet available. However, extensive reports exist on GSTs from other insect species, such as *D. melonogaster* (Adams *et al,* 2000), *Ae. aegypti* (Lumjuan, 2005) and *An. gambiae* (Holt *et al,* 2002) whose genome sequences are accessible from databases. In particular, the *An. gambiae* Epsilon class GST gene cluster has been well characterised in terms of cytological location, structure and genomic organisation (see references in Chapter 1). The eight GST genes isolated from *An. arabiensis* in this study were categorised as Epsilon class GSTs based on their amino acid sequence identity and the phylogenic relationship to *An. gambiae* GST genes (Ortelli *et al,* 2003; Ding *et al,* 2003). The tree in Figure 3.5 shows the relationship of *An. arabiensis* class Epsilon GSTs to the other insect GSTs. Eight members were found in *An. arabiensis* as in *An. gambiae*. The fruit fly contains 10 Epsilon GSTs, but clear orthologs cannot be detected between these genes and those from the mosquito. This result strongly supports the idea that the Epsilon GST genes in *Drosphila* and *Anopheles* have radiated independently (Ranson *et al,* 2002). As expected, the Epsilon GST genes in *An. arabiensis* and *An. gambiae* are phylogenetically closer to one another than to the seven orthologs in *Ae. aegypti*. The other insect-specific GST class, the Delta class, has 12 and 9 members in *An. gambiae* and *Ae. aegypti* respectively. Some of the Delta GST genes have been identified in *An. arabiensis* (data not shown).The relationship between GSTs in *An. arabiensis, An. gambiae* and *Ae. aegypti* cannot be fully explored until the full

complement of GST classes have been identified in *An. arabiensis* genome. In the present study, the transcripts of three of the *An. arabiensis* GSTs, *GSTe1*, *GSTe2* and *GSTe4*, in developmental stages of DDT-resistant KGB and susceptible MAT strains were quantified. All the three Epsilon GSTs were over-expressed in the resistant KGB compared to the MAT strain. However, the expression level of each Epsilon GST was variable within strains and developmental stages and in most cases the expression was highest in the larval life stage. For instance, the normalised copy number of class Epsilon GST transcript relative to *SP7* in KGB-R, ranged from 2.426 (*GSTe2*) to 0.154 (*GSTe4*) in larvae compared to 0.938 to 0.135 in adults.

A significant increase in *GSTe2* transcript copy was observed in both the susceptible MAT and resistant KGB strains following exposure to DDT, permethrin and H_2O_2. Exposure to DDT resulted in a 2.1 - fold increase in *GSTe2* expression above constitutive level in the KGB strain (Figure 5.2). Ding *et al,* (2003) showed that *GSTe2* was constitutively over-expressed in DDT-resistant *An. gambiae* and elevation in the activity of the enzyme was associated with metabolic resistance (Ranson *et al,* 2001). The current study showed that *GSTe2* was over-expressed in the KGB relative to the MAT strains and exposure to DDT resulted in significant increase in expression of the gene in both strains.

DDT is an effective inducer of GSTs and P450s in insects. Brandt *et al,* (2002) have shown that both *Cyp61* and *Cyp612d1* were constitutively up-regulated in a DDT-resistant line of *Drosophila* but only *CYP12d1* was up-regulated by induction with DDT. Their findings suggest co-over-expression and differential induction of adjacent cytochrome P450 genes in association with DDT resistance. In this study, *An. arabiensis GSTe2* was up-regulated by exposure to DDT but the inducible expression of the remaining seven genes in the Epsilon class cluster has not been investigated. The expression of *GSTe2* was also inducible by permethrin (2.3 fold) in the MAT and (1.4 fold) in the KGB strains. The expression of the orthologous gene in *An. gambiae* was shown to be inducible by permethrin (David, unpublished data). Vontas *et al,* (2005) showed that exposure to permethrin resulted in differential regulation of many genes in a pyrethroid-resistant RSP *An. gambiae* strain. Although GSTs were not well represented in the microarray used in this experiment, the result showed that many genes are inducible by permethrin.

Therefore, in addition to *GSTe2*, the spectrum of other genes that can be induced by permethrin remains to be investigated in *An. arabiensis*.

A borderline significant increase in *GSTe2* p = 0.029 and 0.005 was observed following exposure to H_2O_2 in the MAT and KGB strains respectively. This result agreed with a previous report that the expression of *GSTe1* and *GSTe2* in *An. gambiae* were inducible by H_2O_2 (Ding *et al,* 2005). However, in *An. gambiae* the two genes were induced only in the DDT-resistant strain, but *GSTe2* was induced in both the KGB and MAT strains in *An. arabiensis*. Oxidative stress is induced by exposure to many insecticides. Peroxidase catalyses the conversion of H_2O_2 generated by oxidative stress into water, thereby protecting the cells from damage. Hitherto, the Drosophila Sigma Dm *GSTs1-1* and *An. gambiae* Epsilon *GSTe1* genes have been reported to confer peroxidase activity (Singh *et al*, 2001; Ortelli *et al,* 2003). But it is possible that the *GSTe2* in *An. arabiensis* confers resistance to insecticides both by direct insecticide metabolism and by moderating the effects of insecticide - induced oxidative stress. The *AaGSTe2* in *Ae. aegypti* possess both DDT dehydrochlorinase and peroxidase activities (Lumjuan *et al,* 2005) but *An. gambiae GSTe2* doesn't and *An. arabiensis* is much closer to *An. gambiae* than to *Ae. aegypti.* However, whether the Epsilon *GSTe2* gene in *An. arabiensis* posses these activities remains to be confirmed.

This study reveals that expression of *GSTe2* in *An. arabiensis* is regulated by xenobiotics and is associated with DDT resistance. There are other well documented cases of xenobiotic-inducible genes that are constitutively over-expressed in insecticide-resistant strains. Le Goff *et al,* (2006) showed that eleven *Cyp* genes, including *Cyp6a2*, *Cyp6a8* and *Cyp12d1*, and three glutathione S-transferases (GST) genes were significantly induced by phenobarbital. The three *Cyp* genes have been previously associated with insecticide resistance in laboratory-selected strains.

Different hypotheses have been proposed to explain the link between induction and resistance and the possible underlying mechanisms that are involved. For instance, it was proposed that both metabolic resistance and induction were associated with the production of different forms of GSTs (Ottea and Plapp, 1984). Terriere and Yu (1974) suggested that higher expression in resistant flies was likely due to P450

gene amplification, but they rejected induction as a factor in the development of resistance in the field. It was also suggested that the same regulatory (*trans* - acting) gene may be involved in both induction and biochemical resistance Terriere, (1983) and Plapp (1984) further proposed that a receptor involved in xenobiotic induction may be altered in resistant insects.

Mutations in *cis* or *trans*-regulatory elements and post-transcriptional regulation leading to enhanced stability of the transcript, are possible mechanisms causing over expression of GSTs in resistant insects (Ranson and Hemingway, 2005). To delineate the transcriptional mechanisms of the Epsilon GST cluster, putative promoter regions of Epsilon GSTs from the two strains and field-collected specimens of *An. arabiensis* were sequenced. Sequence analysis of the regulatory regions of the Epsilon GST cluster in *An. arabiensis* showed that the promoters of *GSTe1* and *GSTe2* genes encompassed several core promoter elements, including a TATA box, an initiator GC box and a CAAT box (Figures 5.5, 5.6). These basal promoter elements are very similar to those in *An. gambiae* (Ding *et al,* 2005). In *An. gambiae* the over-expression of *GSTe2* is partly due to the deletion of the two adenosines in the promoter of *GSTe2* gene in the DDT-resistant strain (Ding *et al,* 2005). The number of adenosines in the promoter of *GSTe2* in the KGB strain is identical to that in the resistant strain in *An. gambiae*, but the MAT and field strains each had one less residue at each. The significance of these AA-indels in *An. arabiensis* needs to be determined by site, mutagenesis and functional studies. Mutations in cis-acting elements have been associated with insecticide resistance in other species (Dombrowski *et al,* 1998). For example, genetic changes in upstream regulatory region of *Cyp6a2* were hypothesised to affect a putative negative *cis*-acting element, which failed to bind to its relative repressor resulting in over-expression of the gene (Dombrowski *et al*, 1998). Scott (1999), also reported that a *cis*-acting mutation may affect the promoter activity of *Cyp6d1* in houseflies.
Trans-regulatory elements also regulate the expression of GST genes. In *Ae. aegypti* it has been proposed that the regulation of GST is controlled by a *trans*-regulatory element, which generally suppressed the expression of this gene. The alteration of this element increases the expression of GST-2 in a DDT-resistant strain (Grant and Hammock, 1992). Post-transcriptional processing is also involved in the regulation of GSTs via mRNA stability. Enhanced mRNA stability increases the expression of

human *GSTpi* class (Jhaveri *et al,* 1997; Moffat *et al,* 1997). In insects the increase in *DmGSTd21* in *D. melamogaster* is influenced by the stabilisation of *GSTd21* mRNA (Tang and Tu, 1995).

6.3 Field implications of the findings

This study was based entirely in the laboratory, using populations which had been colonised for varying lengths of time. Colonisation provides an opportunity to genetically standardise the test material and the test condition. A lack of genetic variability may be advantageous in investigating a particular resistance mechanism but may also mean that the results obtained in the laboratory do not reflect the situation in the field where the population is inherently heterogeneous (Roush and Mckenzie, 1987) However, various aspects of the laboratory study may be useful in predicting the effect of an increase in frequency of a resistance gene in the field (Penilla *et al,* 1998). In the current study, *An. arabiensis* has been selected for low levels of resistance to DDT and the change in frequency of resistance phenotypes determined. If the laboratory studies result in the identification of particular alleles responsible for resistance, then this information can be informative for control programmes. The obvious concern regarding the spread of resistance has necessitated the establishment of a technical network "The African Network on Vector Resistance (ANVR) to build national capacities for detecting, monitoring and managing vector resistance to insecticides (ANVR, 2002). In this regard the data generated from this study, particularly on the *An. arabiensis* Epsilon GSTs and their potential involvement in DDT resistance in this malaria vector, may ultimately be utilized in control and monitoring programmes. For example, Hargreaves *et al*, (2003) have biochemically detected increased GST activity in the DDT-resistant populations of *An. arabiensis* in Kwazulu Natal in South Africa, but the molecular mechanisms underlying the GST-based DDT resistance is not yet known. In Zimbabwe the WHO bioassay susceptibility tests results showed 81.8% and 70% mortality 24-hour post-exposure to DDT in *An. arabiensis* samples from a sentinel site in Gokwe (Masendu, 2002), but the resistant genotypes have not been determined.

During this study, analysis of the *GSTe2* promoter revealed differences in the number of adenosines, six in the KGB and seven each in MAT and field strains of *An. arabiensis*. Ding *et al,* (2005) have reported similar genetic changes in homologous regions in ZANU and Kisumu strains of *An. gambiae*. These genetic changes, if found to be fixed in field populations of *An. arabiensis*, may be explored further to develop allele-specific PCR assays. These assays can be used to screen field populations and to detect the genes responsible for metabolic resistance to DDT in populations of both *An. arabiensis* and *An. gambiae*. The over-expression of *GSTe2* in the KGB-R indicates positive correlation of the gene expression with DDT selection pressure. Highly adaptive resistance genes, such as *GSTe2* or *kdr*, that are expected to confer some fitness gains to the carriers in areas of intense selection pressure may be powerful markers to detect events of introgression between genetically isolated populations (Slotman *et al,* 2006). No *kdr* mutation was detected in the populations of the two laboratory strains of *An. arabiensis* during this study. However, recent reports of the presence of *kdr* mutation in *An. arabiensis* in Burkina Faso (Diabate *et al*, 2004) Uganda (Pinto *et al,* 2006), Tanzania (Kulkarni *et al*, 2006) and SENN colony of Sudan origins (Matambo *et al,* 2007) is of great importance at both fundamental and applied levels. Studies on the genetic structure of populations of *An. arabiensis* in some regions in Africa suggested extensive gene flow among populations (Besanky *et al*, 1994; Petrarca *el al*, 2000; Wondji *et al*, 2005). This indicates the strong possibility of the spread of the *kdr* mutation with time in population of *An. arabiensis* across the continent.

The use of insecticides such as pyrethroids and DDT, to which resistance developed in one area, would then be less effective with time in other areas. The data of Diabete *et al,* (2004) has illustrated the practical need for identifying resistance mechanisms that are operating in sympatric populations of *An. arabiensis* and *An. gambiae*. They showed that the resistant segments of the populations of the two sibling species are spatio-temporally distributed in a cotton growing area with strong insecticidal selection pressure in Burkina Faso. The proportion of *An. arabiensis* and the molecular M-form of *An. gambiae* have increased from 2% to 25% and from 3% to 75% respectively. Both were susceptible to pyrethroids and DDT and lacked the *kdr* mutation. However, during the rainy season, the S-form was predominant in the cotton growing areas and resistant to pyrethroid and DDT. The *kdr* mutation

was observed at high frequency in the S-form but not in the M-form, nor in *An. arabiensis*. These data suggest that the different segments of the population experience different selection pressures and may develop different levels of resistance to the insecticides. The rate of the spread of the R gene and its impact on the control of the various populations in the area may also be different. Being aware of this will enable workers in vector control to design a species-specific strategy for resistance management. Considering the relatively high adaptability of *An. arabiensis* to human environments (Coluzzi *et al,* 1979) and the potential explosion in its populations due to increasing drought in most African countries (Lindsay *et al*, 1991) as a consequence of global warming, more research effort needs to be focussed in finding effective methods for controlling this important malaria vector in Africa.

6.4 Further studies

Further biochemical studies using synergists for the various classes of metabolic enzymes would confirm the role of GSTs and or esterases in conferring DDT resistance. The synergist/insecticide method was used to demonstrate presence of both DDT and non-DDT dehydrochlorinase resistance mechanisms in the selected IAN and G1 colonies of *An. gambiae* and *An. arabiensis* respectively (Hemingway, 1981). Studies on DDT metabolism to compare DDT dehydrochlorinase activity between mosquitoes in the selected and parental lines would confirm the involvement of GSTs in DDT resistance in the two laboratory strains of *An. arabiensis*. Since the KGB and MAT strains originated from the same geographical zone (South African region), any difference in DDTase activity between the strains could be attributed to difference in their resistance status (Kimura and Brown, 1964). Metabolic studies were attempted to measure the conversion of DDT to DDE by the enzymes in crude homogenate of mosquitoes from the parental and selected colonies MAT strain. However, the chromatograms of the analysis in all the samples showed peaks that did not correspond to the standard DDE peaks (data not shown). This suggest that no DDE was produced either in the resistant or susceptible mosquito homogenates, or if present were in quantities below the resolution of the HPLC. Furthermore studies of recombinant *GSTe2* from the KGB – R strain would verify the ability of *GSTE2* to catalyse the detoxification of DDT.

A more detailed investigation on the transcription of Epsilon GST cluster in *An. arabiensis* would be required in order to understand the complex regulation of this gene family. Analysis of the promoter regions for *An. arabiensis GSTe4*, *GSTe3* and *GSTe8*, particularly the 1260 bp intergenic space between *GSTe6* and *GSTe8* which appears to encompass a functional promoter in *An. gambiae*, may give insight in the mechanisms controlling these genes in *An. arabiensis*. For example, identification of the promoter elements in the region could confirm tentatively, if the transcriptional orientation of GSTe6 is opposite to the neighbouring GST genes as was found in *An. gambiae* (Ding *et al*, 2003).

To study the functions of putative transcription factor binding sites identified in the regulatory regions of *An. arabiensis GSTe1* and *GSTe2* genes, experiments such as promoter activity assays and site – directed mutagenesis studies could be performed (Hawkes and Hemingway 2002; Ding *et al*,2005).

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